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PLANT PHYSIOLOGY

VOLUME 24 JANUARY 1949 NUMBER 1

COPPER ENZYMES IN ISOLATED CHLOROPLASTS. POLYPHENOLOXIDASE IN *BETA VULGARIS*

DANIEL I. ARNON*

(WITH EIGHT FIGURES)

Received August 4, 1948

The chloroplast, as the seat of chlorophyll pigments in plants, occupies a unique position in the economy of the green cell. In recent years there has been a renewed interest in the reactions and properties of chloroplasts as a result of the work of HILL (**11, 12**) and HILL and SCARISBRICK (**13, 14**) who demonstrated that the reaction characteristic of photosynthesis in green plants, the evolution of oxygen, occurs in appreciable quantities in isolated chloroplasts under the influence of light and in the presence of suitable oxidants (**2, 7, 8, 26**).

In the course of an investigation of oxygen evolution by isolated chloroplasts it was deemed important to explore their enzymatic composition. Of special interest were considered enzymes capable of participating in oxidation-reduction reactions, and more particularly, those localized principally, if not entirely, in the chloroplasts. This paper presents evidence that a copper enzyme, polyphenoloxidase (otherwise known as tyrosinase or catecholase), is localized in the chloroplasts of spinach beet (chard), *Beta vulgaris*.

Methods

GROWING OF PLANTS.—Seeds of spinach beet were germinated and grown in sand in flower pots in a greenhouse, heated to prevent the temperature from falling below freezing in winter. The pots were watered with a nutrient solution of the following composition (made with local tap water): KNO_3—0.01M; $Ca(NO_3)_2$—0.002M; $NH_4H_2PO_4$—0.002M; $MgSO_4$—0.002M; and A4, a combination of the micronutrients Mn, B, ~~Ca~~ Cu, Zn

* Fellow of the John Simon Guggenheim Memorial Foundation at the University of Cambridge, England. Permanent address: Division of Plant Nutrition, University of California, Berkeley.

(1). Iron was supplied with each application of the nutrient solution at the rate of 0.5 cc. of 0.5 per cent. ferrous sulphate solution to a liter of nutrient solution. The nutrient solution was applied at variable intervals depending on the weather: in the more favorable periods approximately every other day. Following five successive applications of nutrient solution, the cultures were thoroughly leached with tap water to remove excess salt, just prior to the next application of nutrient solution.

CHLOROPLAST PREPARATIONS.—The chloroplast material used throughout consisted not of intact chloroplasts but rather of chloroplast fragments which were obtained by the preparatory technique to be presently described. Preparations of this type are sometimes referred to as "grana". If that term be appropriately reserved for the naturally occurring dense bodies in chloroplasts, as recently demonstrated with the electron microscope by GRANICK & PORTER (10), the chloroplast fragments used in this investigation may be regarded as aggregates of grana embedded in the matrix or "stroma".

The chloroplast fragments were prepared by a procedure similar to that of ARONOFF (2). Leaves were picked from plants grown in sand cultures and placed in cold tap water. After a thorough washing in running cold water, they were put in moisture-proof pliofilm bags and kept from one half to several hours in a refrigerator. The turgid leaves were torn by hand into small shreds and ground for three minutes in a Waring Blendor with either M/15 potassium phosphate buffer or glass distilled water. About 50 grams of laminae (the midrib and large veins were discarded) were blended with 60 cc. of fluid. The speed of the Blendor was regulated by means of a variable voltage transformer to avoid foaming.

The Blendor mash was strained through cheesecloth and immediately filtered through a small plug of absorbent cotton placed in a glass funnel. The residue was discarded and the filtrate was centrifuged in an angle centrifuge for 1 minute at approximately 3000 r.p.m. to remove intact chloroplasts and such larger particles as still remained after straining through cheesecloth and filtering through cotton. The supernatant fluid from the brief centrifugation was a dark green fluid containing, as disclosed by microscopic examination, chloroplast fragments with few intact chloroplasts. The residue which included the intact chloroplasts was discarded. The supernatant was decanted and centrifuged for 20 minutes at about 4° C. in an International Refrigerated Centrifuge at a speed corresponding to approximately 25,000 *g*.

The high speed centrifugation brought about a sedimentation of the chloroplast fragments, leaving a pale yellowish-green supernatant which contained only a minute amount of chlorophyll. The supernatant, henceforth designated as the cytoplasmic fluid, was decanted. The residue was rinsed quickly in M/15 phosphate buffer at pH 6.5 to remove the adhering cytoplasmic fluid, and then resuspended in a small volume of M/15 phos-

phate buffered at pH 6.5 with the aid of a glass stirring rod or a small glass spatula and filtered again through a small wad of absorbent cotton. The last operation was combined with gentle pressing, by means of a glass rod, of the chloroplast suspension against the wad of cotton—a procedure found effective in breaking up lumps. The resuspended chloroplast fragments which will henceforth be designated as the chloroplast suspension, contained between forty and fifty times as much chlorophyll as the cytoplasmic fluid.

DETERMINATION OF CHLOROPHYLL.—The procedure for chlorophyll determination was based on the work of MACKINNEY (**21**) on the absorption of light by aqueous acetone (80%) extracts of chlorophyll. The concentrations of chlorophyll a and b were determined by measuring in a 10 mm. cell, the density of 80% acetone chlorophyll extracts with a Beckman spectrophotometer at 663 and 645 mμ and setting up simultaneous equations using the specific absorption coefficients for chlorophyll a and b as given by MACKINNEY (**21**).

(i) $D_{663} = 82.04\ C_a + 9.27\ C_b$

(ii) $D_{645} = 16.75\ C_a + 45.6\ C_b$

where C_a, C_b are grams per liter of chlorophyll a and b respectively, and D the density values at the respective wavelengths as obtained on the Beckman spectrophotometer. From equation (ii):

(iii) $C_a = \dfrac{D_{645} - 45.6\ C_b}{16.75}$, substituting in (i) and solving for C_b we obtain

(iv) $C_b = 0.0229\ D_{645} - 0.00468\ D_{663}$; substituting this expression for C_b in (ii) and solving for C_a:

(v) $C_a = 0.0127\ D_{663} - 0.00269\ D_{645}$

Hence total chlorophyll, C, in grams per liter:

(vi) $C = C_a + C_b = 0.0202\ D_{645} + 0.00802\ D_{663}$ or expressing total chlorophyll, C, in terms of milligrams per liter:

(vii) $C = 20.2\ D_{645} + 8.02\ D_{663}$

To provide a rough check for the determination of total chlorophyll the light absorption data for chlorophyll a and b (**21**) were plotted and the curves were found to intersect at $\lambda = 652$ mμ. By extrapolation, the value of the specific absorption coefficient for this wavelength was found to be 34.5. Another equation was therefore set up for total chlorophyll:

(viii) $D_{652} = 34.5\ C + 34.5\ C_b = 34.5\ (C_a + C_b)$

hence (ix) $C = (C_a + C_b) = \dfrac{D_{652}}{34.5}$

or expressed as mgs. per liter:

(x) $C = \dfrac{D_{652} \times 1000}{34.5}$

The following example will illustrate the procedure used for chlorophyll determinations; 0.5 cc. of the chloroplast suspension was shaken in a glass stoppered volumetric cylinder with 4.5 cc. of water and 20 cc. of acetone. The suspension was filtered and the density values for the respective wave-

lengths were determined on a Beckman spectrophotometer to be:

$$D_{645} = 0.187$$
$$D_{663} = 0.506$$
$$D_{652} = 0.275$$

Substituting the first two values into equation (vii) and multiplying by the dilution factor:

$$C = (20.2 \times 0.187 + 8.02 \times 0.506) \times \frac{25}{1000 \times 0.5} = 0.39 \text{ mgs. chlorophyll/cc.}$$

Similarly, by substituting for D_{652} in equation (x):

$$C = \frac{0.275 \times 1000}{34.5} \times \frac{25}{1000 \times 0.5} = 0.399 \text{ mgs. chlorophyll/cc.}$$

The example given illustrates the generally good agreement between the two values obtained for total chlorophyll concentration. It is clear, however, that equation (vii), which is based on the selection of more favorable wave lengths for precise measurement, is more trustworthy than equation (x).

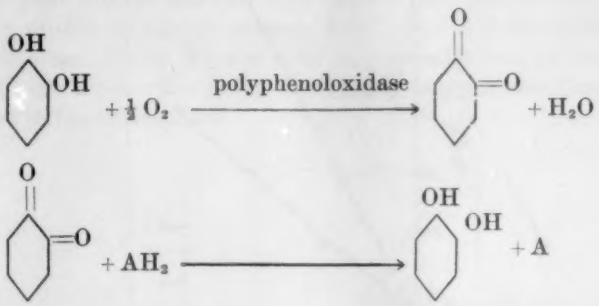
The size of the aliquot used for chlorophyll determination was governed by the chlorophyll content. Generally 0.5 cc. of the chloroplast suspension or 5 cc. of the cytoplasmic fluid in 25 cc. of 80 per cent. acetone were found convenient.

For the determination of enzymatic activity generally 2 cc. of either the chloroplast suspension or the cytoplasmic fluid was placed in each reaction vessel. In the case of the chloroplast suspension this corresponded to a chlorophyll content of between 0.7 and 2.0 mgs.

MEASUREMENTS OF POLYPHENOLOXIDASE ACTIVITY.—All measurements of enzymatic activity were made manometrically at 20° C. using conical vessels of approximately 16 cc. volume. Catechol was used in the estimation of polyphenoloxidase activity, not as a substrate, however, but as a catalyst in the oxidation of ascorbic acid. The use of small quantities of catechol in the measurement of polyphenoloxidase activity was demonstrated by KUBOWITZ (**18**) in the oxidation of hexose monophosphate in the presence of its dehydrogenase and coenzyme II. KEILIN and MANN (**16**) have shown that although polyphenoloxidase does not oxidize ascorbic acid directly, the oxidation proceeds very rapidly in the presence of small amounts of catechol. This principle was also used by MILLER and DAWSON (**22**) in developing the chronometric method for measuring polyphenoloxidase activity. The catechol is enzymatically oxidized to the *o*-quinone by atmospheric oxygen; the *o*-quinone in turn is almost instantaneously reduced back to catechol by the ascorbic acid. The reaction comes to completion when all of the ascorbic acid has been oxidized.

The substrate mixture used consisted of 0.02 mgs. catechol and from 1.2 to 2.4 mgs. of ascorbic acid. The catechol was introduced to the side-arm and tipped into the main vessel at zero time. The total volume of the reaction mixture was 3 cc. Except as noted otherwise, the gas phase

was air; to absorb CO_2, the center well contained 0.2 cc. of 10% KOH and a small strip of filter paper. In measuring cyanide inhibition the center well fluid consisted of a mixture of KCN and KOH in the proportions given by ROBBIE (**25**). The reaction mixture was prepared and made to volume with M/15 phosphate buffer of pH 6.5.



where AH_2 and A stand for ascorbic and dehydroascorbic acid respectively.

Half of the bottom of the constant temperature bath was made of plate glass. In testing the effect of light on the reversibility of carbon monoxide inhibition the reaction vessels were illuminated from below by a 500 watt condensed filament bulb placed in a specially designed metal reflector. The light intensity in the bath as measured by a Weston lightmeter was approximately 16,000 lux.

Results

LOCALIZATION OF POLYPHENOLOXIDASE ACTIVITY.—In a preliminary experiment it was found that the addition of catechol to a crude leaf mash of spinach beet plant was followed by a rapid oxygen uptake. This suggested the presence of a polyphenoloxidase in the leaves and it became of interest to determine whether this enzyme is found in the chloroplasts or in the cytoplasmic fluid, obtained after the separation of chloroplasts and their fragments.

As stated earlier, the method selected for measuring polyphenoloxidase activity was the oxidation of ascorbic acid with the addition of catalytic amounts of catechol to act as an oxygen carrier: oxidizable by the enzyme and reducible by the ascorbic acid. There was thus at no time a high concentration of the possibly toxic *o*-quinone in the reaction mixture (**18, 23**). Curve II in figure 1 shows that the leaf mash was capable of oxidizing ascorbic acid at a slow rate without the addition of catechol. This oxidation was, however, greatly accelerated by the addition of 0.02 mgs. of catechol (Curve I, fig. 1).

Upon separation of the chloroplast fragments from the cytoplasm it was found that the polyphenoloxidase activity was localized in the chloro-

plasts (fig. 2). Rapid uptake of oxygen and oxidation of ascorbic acid followed the addition of catechol to the chloroplast suspension (Curve I). Without the addition of catechol (Curve II) or with catechol but without

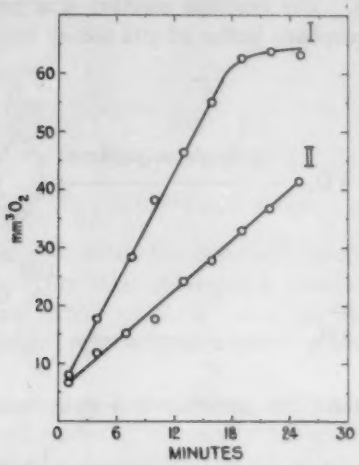


FIG. 1. Effect of added catechol on oxidation of ascorbic acid by leaf mash (supernatant after 1 minute centrifugation as described under Methods). Curve I—0.02 mgs. catechol in 0.2 cc. phosphate buffer tipped in at zero time to reaction mixture; Curve II—no catechol added. Reaction mixture: 2 cc. of leaf mash containing 0.7 mgs. chlorophyll, 1.2 mgs. ascorbic acid in 0.6 cc. phosphate buffer, M/15 phosphate buffer (pH 6.5) as needed to make a total volume of 3 cc. 0.2 cc. of 10% KOH and filter paper in center well.

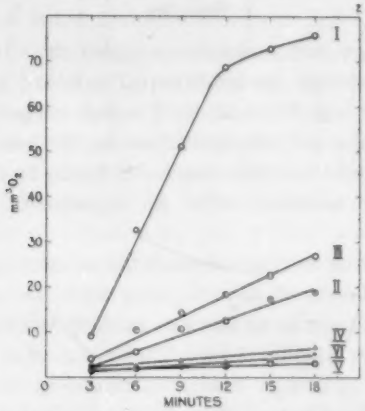


FIG. 2. Polyphenoloxidase activity of chloroplast suspensions and cytoplasmic fluid. Curve I—chloroplast suspension, catechol and ascorbic acid. Curve II—chloroplast suspension and ascorbic acid but no catechol. Curve III—chloroplast suspension, catechol but no ascorbic acid. Curve IV—cytoplasmic fluid, catechol and ascorbic acid. Curve V—cytoplasmic fluid, ascorbic acid but no catechol. Curve VI—cytoplasmic fluid, catechol but no ascorbic acid. Approximately 1 mg. of chlorophyll per vessel in chloroplast suspensions and 0.02 mgs. of chlorophyll in cytoplasmic fluid. Conditions not specified were the same as given in the legend of figure 1.

ascorbic acid (Curve III) the oxygen uptake was greatly reduced. Such residual enzymatic activity as was observed under these two latter conditions was probably due to substances naturally occurring in the chloroplasts which were capable of serving as substrates.

In contrast to the chloroplast suspensions, the addition of catechol, with (Curve IV) or without ascorbic acid (Curve VI) was without effect on the oxygen uptake by the cytoplasmic fluid. Nor did the addition of ascorbic acid alone (Curve V) result in an increased rate of oxidation by the cytoplasmic fluid. The polyphenoloxidase activity was thus found to be localized in the chloroplasts.

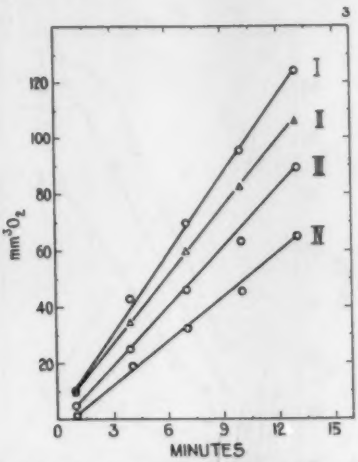


FIG. 3. Effect of cyanide on polyphenoloxidase activity of chloroplast suspensions. Curve I—control; Curve II—4.6×10^{-5}M CN; Curve III—10^{-4}M CN; Curve IV—4.6×10^{-4}M CN. 2 mgs. of ascorbic acid in all reaction mixtures. Center well contains KCN–KOH mixture. Conditions not specified were the same as given in the legend for Curve I of figure 1.

EFFECT OF INHIBITORS.—Consistent with the well established copper-protein nature of polyphenoloxidase (**16, 18, 19**), the activity of the enzyme in the chloroplast suspensions was inhibited by cyanide and carbon monoxide, the latter being insensitive to light (**15**). The enzyme was also inhibited by a more specific copper poison, sodium diethyldithiocarbamate (**20**). The effects of cyanide on the polyphenoloxidase activity of the chloroplast suspension are shown in figure 3, the inhibition by diethyldithiocarbamate in figure 4, and the inhibition by carbon monoxide in figure 5. As shown by Curve II of figure 5, light did not reverse the inhibition of enzyme activity by carbon monoxide.

IS THE LOCALIZATION OF POLYPHENOLOXIDASE ACTIVITY IN THE CHLOROPLASTS TO BE ATTRIBUTED TO PREPARATORY TECHNIQUE?—This question deserves careful consideration in attempts to identify the original locus of an enzyme found in the mash of leaves. The procedures used for disintegrating

leaves were necessarily drastic and resulted in disruption of cytoplasmic bodies including the chloroplasts. The persistence, however, of an enzyme in the resulting fragments, in this instance in the chloroplast suspension, and its absence from the cytoplasmic fluid, is under the circumstances regarded as strong presumptive evidence that the chloroplasts were the original site of the enzyme in the leaf. Conversely, were the enzyme to be found either partly or wholly in the cytoplasmic fluid, the possibility that it passed there following the fragmentation of chloroplasts could not have been easily excluded. Further experiments were carried out to test the conclusion about the localization of polyphenoloxidase in the chloro-

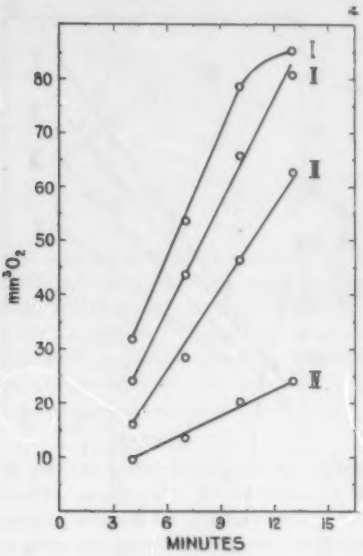


FIG. 4. Effect of sodium diethyldithiocarbamate (DDC) on polyphenoloxidase activity of chloroplast suspensions. Curve I—control; Curve II—10^{-4}M DDC; Curve III —5×10^{-4}M DDC; Curve IV—10^{-3}M DDC. 1.5 mgs. of chlorophyll in all reaction mixtures. Conditions not specified were the same as given in the legend for Curve I of figure 1.

plasts, by modifying the experimental procedures in several respects, giving special attention to differences in technique which might have accounted for such divergent reports as were found in the literature.

a) The addition of potato polyphenoloxidase to leaf mash of spinach beet: the possibility was considered that if the polyphenoloxidase in spinach beet leaf were originally present in the cytoplasmic fluid, then the high-speed centrifugation used in separating the chloroplast fragments might have sedimented the enzyme as well. This point was tested by adding a crude potato polyphenoloxidase to the spinach beet leaf mash and then proceeding with the separation of the chloroplast fragments. The potato

polyphenoloxidase was prepared by a procedure similar to that of Szent-Györgyi as given by KEILIN (**15**).

A preparation of spinach beet leaf mash was divided into two batches. To one a solution of potato polyphenoloxidase in M/15 phosphate buffer (pH 6.5) was added, whereas the other batch served as a control. The

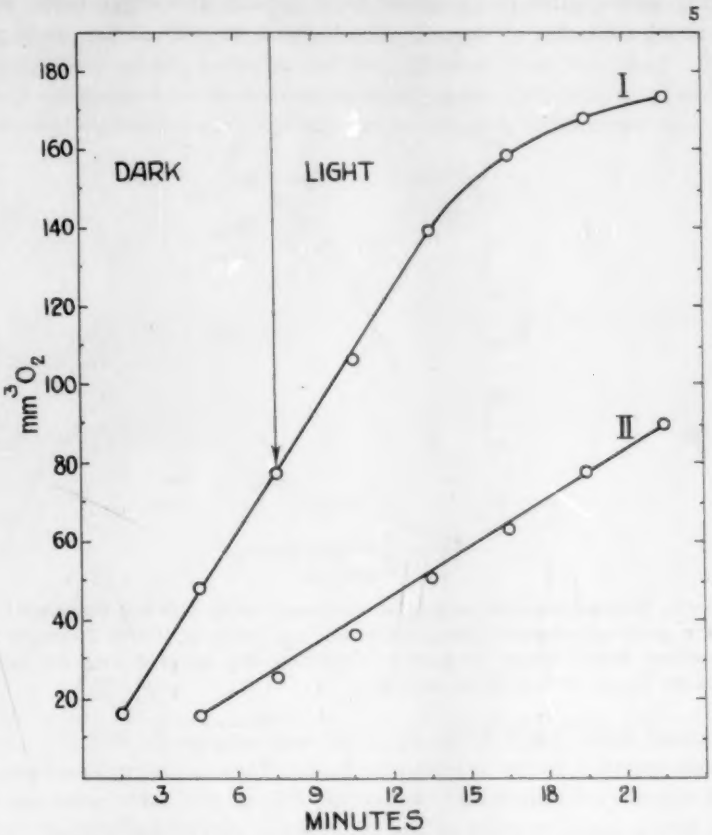


FIG. 5. Effect of carbon monoxide on polyphenoloxidase activity of chloroplast suspensions. Curve I—control in air; Curve II—90% CO—10% O_2. The turning on of light at 7.5 minutes did not reverse the inhibition by carbon monoxide. 2.2 mgs. of chlorophyll and 2.4 mgs. of ascorbic acid in each of the reaction mixtures. Conditions not specified were the same as given in the legend for Curve I of figure 1.

chloroplast fragments were then removed from the two batches of leaf mash, by high-speed centrifugation as previously described. The remaining cytoplasmic fluids were tested for polyphenoloxidase activity. The results, presented in figure 6, show that the added potato polyphenoloxidase was recovered in the cytoplasmic fluid. The activity of the polyphenoloxidase of the chloroplast suspension prepared from the batch of

leaf mash to which the potato enzyme was added was not enhanced. The conclusion was therefore drawn that the polyphenoloxidase activity of the chloroplast suspension was not to be attributed to sedimentation during the high-speed centrifugation.

b) Effect of pH of extracting buffer. WARBURG and LÜTTGENS (**26**), working with similar preparations from spinach and sugar beets, attributed about a quarter of the polyphenoloxidase activity of the crude green

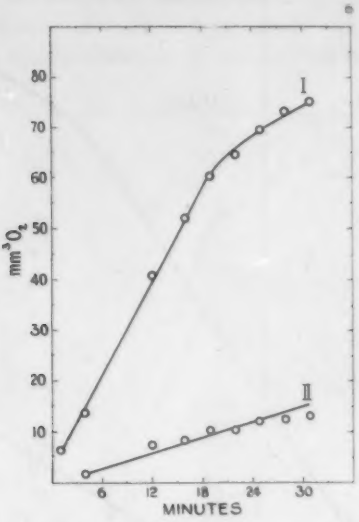


FIG. 6. Polyphenoloxidase activity of cytoplasmic fluids with and without the addition of a crude polyphenoloxidase preparation from potatoes. Curve I—potato polyphenoloxidase added; Curve II—control. Conditions not specified were the same as given in the legend of Curve I in figure 1.

leaf extract made at pH 6.5 to the chloroplast suspension and the remaining three quarters to the cytoplasmic fluid. These authors considered the pH of the extract important. Below pH 6.5, at pH 5 for example, they found that a major portion of the cytoplasmic polyphenoloxidase precipitated and in a subsequent centrifugation was recovered with the chloroplast fragments.

Although the preparations used in this investigation were uniformly made at pH 6.5 it was undertaken to test the effect of hydrogen ion concentration on the distribution of polyphenoloxidase by extracting the chloroplast suspension at the more alkaline pH 7.3. M/15 potassium phosphate buffer of pH 7.3 was used for grinding the leaves and resuspending the chloroplast fragments, the procedures otherwise remaining as previously described. The results presented in figure 7 show that, as with the former preparations, the polyphenoloxidase activity of leaf extracts made at pH 7.3 was also localized in the chloroplasts.

c) **Effect of further disintegration of chloroplast suspensions.** BONNER and WILDMAN (**4**) studied the respiration of spinach leaves and concluded that polyphenoloxidase is their terminal oxidase. Unlike the present findings, however, these authors reported the presence of polyphenoloxidase as confined to the cytoplasm rather than to the chloroplasts. The technique used by WILDMAN and BONNER (**27**) involved grinding the chloroplasts in a Charlotte Colloid mill with resultant disintegration of chloroplasts into chlorophyll-containing granules and non-chlorophyllous "stroma." These authors are aware that their conclusions as to the distribution of enzymes between chloroplasts and cytoplasm can be properly interpreted only with

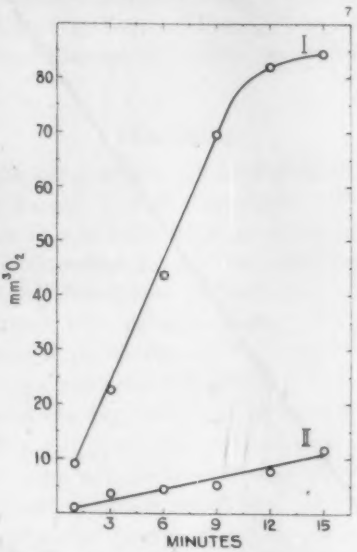


FIG. 7. The polyphenoloxidase activity of chloroplast suspension and cytoplasmic fluid prepared at an alkaline pH. The preparations and the reaction mixtures were made with potassium phosphate buffer (pH 7.3). Curve I—chloroplast suspension containing 1.5 mgs. chlorophyll; Curve II—cytoplasmic fluid. Conditions not specified are the same as given in the legend for Curve I of figure 1.

reference to their own manipulative technique. It was already noted that in the same plant, spinach, WARBURG and LÜTTGENS (**26**) found polyphenoloxidase both in chloroplast fragments and in cytoplasm. The spinach beet used in the present investigation is a chenopodiaceous plant related to sugar beet and spinach.

It seemed desirable in view of the results of BONNER and WILDMAN (**4**) to subject the already disrupted chloroplasts comprising the chloroplast suspensions used in this investigation to further disintegration. This was done by grinding them with glass beads on a Mickle tissue-disintegrating machine. The ground chloroplasts were then separated by high-speed centrifugation and resuspended in buffer as previously described. A micro-

scopic examination of the ground chloroplast suspension disclosed uniform round particles over 1 millimicron in diameter.

Figure 8 shows that this further subdivision of the chloroplast fragments failed to dislodge the polyphenoloxidase. As before, the enzyme

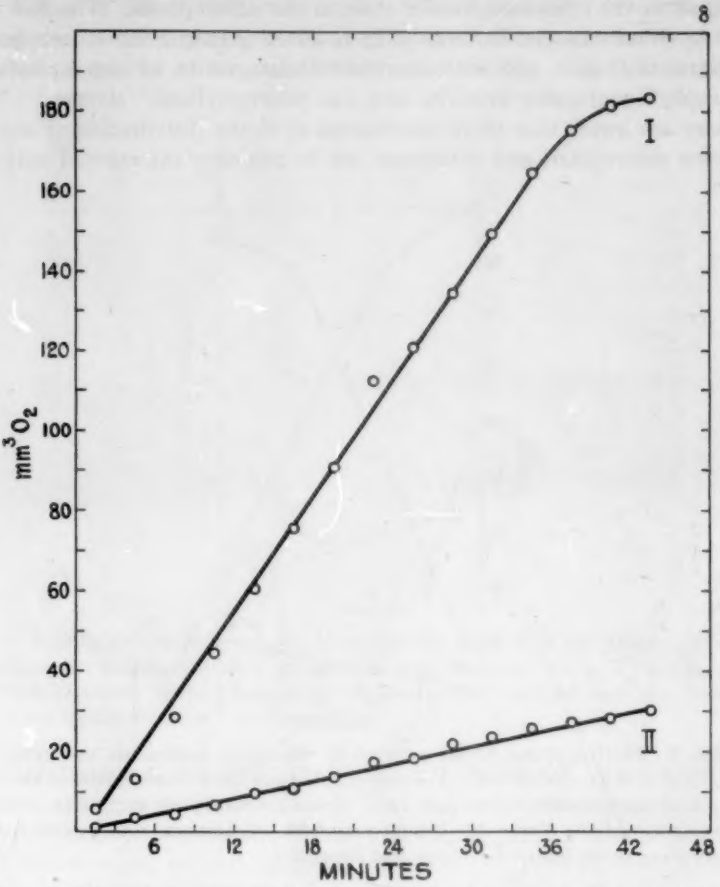


FIG. 8. Effect of further disintegration of chloroplasts on polyphenoloxidase activity. Curve I—disintegrated chloroplast suspension containing 0.8 mgs. of chlorophyll; Curve II—cytoplasmic fluid containing 0.02 mgs. of chlorophyll after removal of disintegrated chloroplast fragments. Reaction mixtures contained 2.4 mgs. ascorbic acid. Conditions not specified were the same as those given in the legend of Curve I in figure 1.

was found in the chloroplast suspension rather than in the cytoplasmic fluid.

TEST FOR LACCASE ACTIVITY OF CHLOROPLAST SUSPENSIONS AND CYTOPLASMIC FLUID.—The finding of polyphenoloxidase (an orthophenolase) activity in chloroplasts raised the question whether another copper protein enzyme,

laccase, which readily oxidizes paraphenols (**17**) occurs in leaf preparations. On *a priori* grounds this was considered unlikely: crude laccase oxidizes ascorbic acid directly (**17**) and it was already shown that neither the chloroplast suspensions nor the supernatant oxidized ascorbic acid directly at an appreciable rate. Furthermore, laccase, though capable of oxidizing catechol, is, unlike polyphenoloxidase, not inhibited by carbon monoxide (**17**). It was already shown, however, in figure 5, that catechol oxidation by chloroplast suspensions was inhibited by CO.

A preliminary test for laccase activity was made by adding 2 mgs. of *p*-phenylene diamine as a substrate to both the chloroplast suspension and the cytoplasmic fluid under conditions described in the legend of figure 1. The results indicated that no increased oxygen uptake resulted from the addition of *p*-phenylene diamine to either the chloroplast suspension or the cytoplasmic fluid.

Discussion

The presented data demonstrate the localization of the polyphenoloxidase of spinach beet leaves in the chloroplasts. The concentration of a copper-protein oxidase in a cellular body so intimately identified with the process of photosynthesis raises at once the question of the possible participation of this enzyme directly or indirectly in one or more of the biochemical reactions linked with photosynthesis. The answer to this question must await further experimentation.

In recent years there has been active interest in polyphenoloxidase as the terminal oxidase in the respiration of the potato tuber (**3, 5, 6**) and spinach leaves (**4, 27**). In Chlorella cells copper "poisons" have been found to inhibit both photosynthesis and respiration (**9**).

Whatever the ultimate position which may be assigned to polyphenoloxidase in the metabolism of plants, it appears certain that this enzyme cannot be of universal importance in the plant kingdom. This view is based on the findings of ONSLOW (**24**) that many, including some of the most common, mono- and dicotyledonous plants do not contain polyphenoloxidase. The elucidation, however, of its rôle in those plants in which the enzyme is found promises to lead to advances in comparative biochemistry through a search for its counterpart in other plants.

Summary

1. A green leaf extract of spinach beet (chard) was fractionated by high-speed centrifugation into a suspension of chloroplast fragments and a "cytoplasmic fluid" practically free of chlorophyll.

2. Polyphenoloxidase activity of the two fractions was tested by using catalytic amounts of catechol with ascorbic acid as substrate, and was found to be localized in the chloroplast suspension.

3. The localization of polyphenoloxidase in the chloroplast suspension was confirmed by using several variants in manipulative technique: ex-

traction of chloroplast fragments at a different pH, further disintegration of the chloroplast fragments, and the addition of a crude potato polyphenoloxidase to the leaf mash.

4. Consistent with the copper-protein nature of the enzyme, its activity in the chloroplasts was inhibited by cyanide, sodium diethyldithiocarbamate and carbon monoxide. The inhibition by carbon monoxide was not reversed by light.

Acknowledgments

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Molteno Institute,
University of Cambridge, England.

LITERATURE CITED

1. Arnon, D. I. Microelements in culture-solution experiments with higher plants. Amer. Jour. Bot. **25**: 322–325. 1938.
2. Aronoff, S. Photochemical reduction of chloroplast grana. Plant Physiol. **21**: 393–409. 1946.
3. Baker, D. and Nelson, J. M. Tyrosinase and plant respiration. J. Gen. Physiol. **26**: 269–276. 1943.
4. Bonner, J. and Wildman, S. G. Enzymatic mechanisms in the respiration of spinach leaves. Arch. Biochem. **10**: 497–518. 1946.
5. Boswell, J. G. Oxidation systems in the potato tuber. Ann. Bot. n.s. **9**: 55–76. 1945.
6. Boswell, J. G. and Whiting, G. C. A study of the polyphenoloxidase system in potato tubers. Ann. Bot. n.s. **2**: 847–864. 1938.
7. Brown, Allan H. and Franck, J. On the participation of carbon dioxide in the photosynthetic activity of illuminated chloroplast suspensions. Arch. Biochem. **16**: 55–60. 1948.
8. French, C. S., Holt, A. S., Powell, R. D., and Anson, M. L. The evolution of oxygen from illuminated suspensions of frozen, dried and homogenized chloroplasts. Science **103**: 505–506. 1946.
9. Green, L. F., McCarthy, J. F., and King, C. G. Inhibition of respiration and photosynthesis in Chlorella pyrenoidosa by organic compounds that inhibit copper catalysis. J. Biol. Chem. **128**: 447–453. 1939.
10. Granick, S., and Porter, K. R. The structure of the spinach chloroplast as interpreted with the electron microscope. Amer. Jour. Bot. **34**: 545–550. 1947.
11. Hill, R. Oxygen evolved by isolated chloroplasts. Nature **139**: 881–882. 1937.
12. Hill, R. Oxygen produced by isolated chloroplasts. Proc. Roy. Soc. London B **127**: 192–210. 1939.

13. HILL, R., and SCARISBRICK, R. Production of oxygen by illuminated chloroplasts. Nature **146**: 61. 1940.
14. HILL, R., and SCARISBRICK, R. The reduction of ferric oxalate by isolated chloroplasts. Proc. Roy. Soc. London B **129**: 238–255. 1940.
15. KEILIN, D. Cytochrome and respiratory enzymes. Proc. Roy. Soc. London B **104**: 206–252. 1929.
16. KEILIN, D., and MANN, T. Polyphenol oxidase, purification, nature and properties. Proc. Roy. Soc. London B **125**: 187–204. 1938.
17. KEILIN, D., and MANN, T. Laccase, a blue copper-protein oxidase from the latex of *Rhus succedanea*. Nature **143**: 23. 1939.
18. KUBOWITZ, F. Über die chemische Zusammensetzung der Kartoffeloxydase. Bioch. Zeits. **292**: 221–229. 1937.
19. KUBOWITZ, F. Spaltung und Resynthese der Polyphenoloxydase und des Hämocyanins. Bioch. Zeits. **299**: 32–57. 1938.
20. MCCARTHY, J. F., GREEN, L. F., and KING, C. G. The substrate specificity and inhibition characteristics of two copper-protein "oxidases." Jour. Biol. Chem. **128**: 455–462. 1939.
21. MACKINNEY, G. Absorption of light by chlorophyll solutions. Jour. Biol. Chem. **140**: 315–322. 1941.
22. MILLER, W. H., and DAWSON, C. R. A new method for the measurement of tyrosinase catecholase activity. Jour. Amer. Chem. Soc. **63**: 3375–3382. 1941.
23. NELSON, J. M., and DAWSON, C. R. Tyrosinase. Adv. Enzym. **4**: 99–152. 1944.
24. ONSLOW, M. W. The principles of plant biochemistry. Cambridge Univ. Press. 1931.
25. ROBBIE, W. A. The quantitative control of cyanide in manometric experimentation. Jour. Cell. Comp. Physiol. **27**: 181–209. 1946.
26. WARBURG, O., and LÜTTGENS, W. Photochemische Reduktion des Chinons in grünen zellen und Granula. Biochimia **11**: 303–322. 1946.
27. WILDMAN, S. G. and BONNER, J. The proteins of green leaves, I. Isolation, enzymatic properties and auxin content of spinach cytoplasmic proteins. Arch. Biochem. **14**: 381–413. 1947.

INFLUENCE OF FERTILIZER TREATMENT ON THE CHEMICAL COMPOSITION OF MOORE PECAN LEAVES DURING NUT DEVELOPMENT[1]

HARALD E. HAMMAR AND JAMES H. HUNTER

(WITH ELEVEN FIGURES)

Received December 6, 1947

Introduction

Chemical and physical changes taking place in pecan nuts during the development and filling period have been reported (**7, 9, 12, 19, 20, 21, 22**) and studies have been made on seasonal changes in the chemical composition of pecan and other tree leaves (**7, 8, 12, 13, 15, 16, 17**). These studies show that large amounts of nitrogen and mineral elements (phosphorus, potassium, calcium, and magnesium) are utilized in the formation of the nut or seed, or accumulated in the leaf tissue during the growing and filling period. Fertilizer treatment has been shown to have considerable influence on the chemical composition of many plants. ALBEN and HAMMAR (**2, 3**) have reported significantly higher phosphorus content in the foliage from pecan trees fertilized with phosphate fertilizer over those receiving no phosphate, and ALBEN (**1**) showed similar trends with potassium. Various chemical elements, such as nitrogen, potassium, calcium, chlorine, boron, and zinc, when supplied to pecan trees, have been found to be reflected in the composition of the foliage.

The rapidity with which chemical changes take place, especially during the early filling period of the pecan nut, has revealed the necessity of maintaining adequate supplies of these nutrients in the plant tissues, especially in prolific varieties such as the Moore, which tend to produce very heavy crops in alternate years. The rapid depletion of mineral elements, especially phosphorus and potassium, in the pecan leaf during the summer months has been observed from leaf samplings made in June and September. Frequently when pecan trees support a heavy crop of nuts, the nuts fail to fill satisfactorily. Observation and limited data on the nutrient levels indicate that poor filling is associated with a depletion of some of the necessary plant food elements. It was hoped that if periodic sampling of pecan leaves during the summer revealed a deficiency of any element at a specific period during the development of the nut, the fertilizer program could be adjusted accordingly.

[1] This paper was presented before the Georgia Section of the American Chemical Society held in Atlanta, November 20, 1947.

Materials and methods

Leaf samples for chemical studies were taken from four trees in each of four differently treated fertilizer blocks in a pecan orchard near Albany, Georgia. The trees were of the Moore variety, about 25 years of age, and each block had received a different fertilizer treatment during the 10 years preceding the sampling in 1945. Fertilizer treatments consisted of: (1) ammonium sulfate; (2) Cyanamid; (3) complete (N-P-K) fertilizer; and (4) no fertilizer. Each fertilizer block consisted of 20 trees. The complete (N-P-K) fertilizer block received 50 pounds per tree of a mixed fertilizer (6–8–4 from 1936 to 1938 and 4–8–4 from 1939 to 1945), and equivalent amounts of nitrogen were applied to the trees receiving ammonium sulfate and Cyanamid. Cover crops, mainly legumes, were grown uniformly each year on the four blocks of trees.

The trees from which the leaf samples were taken were subjected to rather abnormal weather and crop conditions in 1945. The unusually heavy crop load that year seemed to completely devitalize the trees, which may partially account for the striking changes which were found to take place in the composition of the leaves. The relation of this condition to the poor filling of the nuts has recently been reported (**11**) in which the authors showed that seriously devitalized trees may result from a pronounced depletion of phosphorus and potassium in the trees producing heavy crops.

The crop record for these trees for a 10-year period including 1945 has been previously reported (**10**). In order to visualize the crop condition which existed just prior to and including the period the leaf samples were taken, the following data on the yield of nuts is given. The average yield of nuts in pounds per tree in 1945 for each of the fertilizer treatments were: ammonium sulfate, 117; Cyanamid, 103, complete, 124; and no fertilizer, 91; while the yields per tree in 1944 were 0, 0, 6, and 2 pounds, respectively, for the four fertilizer treatments. The heavy crop in 1945 was associated with very poor filling of the kernels, and early deterioration of the leaves.

The four trees from each block from which leaf samples were taken had been selected at random, excepting that no tree was sampled that had a very high or very low yield record. The second leaf from the terminal end of shoots bearing four nuts was taken from at least 50 shoots around the entire periphery of each tree, at six samplings with 3-week intervals from June 11 to September 24, 1945. The leaves were air-dried in the laboratory, and leaflets stripped from the rachises were ground in a Wiley mill to pass a 40-mesh sieve. Before analyzing, the samples were dried overnight in a vacuum oven at a reduced pressure of 25 inches of mercury. The samples for mineral analysis (phosphorus, potassium, calcium, and magnesium) were ashed first under low heat, and finally for three hours in an electric muffle at 550° C. Total nitrogen was determined on separate samples. Official methods of analysis (**4**) were used for all elements except potassium, for which the sodium-cobaltinitrite method of Brown, Robinson, and Browning (**5**) was used. Each determination was made in duplicate.

Results

The mean chemical composition of the leaves from the four trees for each of the four differently treated fertilizer blocks for the six sampling dates is shown in table I. A graphic presentation of the chemical changes taking

TABLE I

THE CHEMICAL COMPOSITION OF MOORE PECAN LEAVES DURING NUT DEVELOPMENT

MEAN PERCENTAGE COMPOSITION—OVEN-DRY BASIS

FERTILIZER TREATMENT	SAMPLING DATES					
	6/11/45	7/2/45	7/23/45	8/13/45	9/4/45	9/24/45
			NITROGEN			
Ammonium Sulfate	2.62	2.56	2.47	2.42	2.22	2.14
Cyanamid	2.57	2.51	2.39	2.32	2.16	2.10
Complete	2.62	2.51	2.42	2.34	2.08	2.06
No Fertilizer	2.54	2.46	2.42	2.26	2.21	2.10
			PHOSPHORUS			
Ammonium Sulfate	0.142	0.120	0.111	0.106	0.082	0.096
Cyanamid	.142	.126	.114	.106	.089	.096
Complete	.158	.130	.129	.129	.105	.105
No Fertilizer	.148	.126	.118	.114	.100	.097
			POTASSIUM			
Ammonium Sulfate	1.077	0.887	0.607	0.566	0.374	0.354
Cyanamid	.978	.764	.516	.462	.354	.347
Complete	1.047	.839	.581	.507	.387	.355
No Fertilizer	.924	.822	.587	.461	.392	.337
			CALCIUM			
Ammonium Sulfate	1.320	1.496	1.662	1.765	1.699	1.812
Cyanamid	1.449	1.625	1.704	1.784	1.715	1.753
Complete	1.629	1.657	1.919	2.005	2.026	2.048
No Fertilizer	1.129	1.250	1.429	1.423	1.430	1.583
			MAGNESIUM			
Ammonium Sulfate	0.419	0.430	0.440	0.454	0.437	0.480
Cyanamid	.434	.433	.431	.412	.397	.438
Complete	.450	.440	.441	.484	.467	.454
No Fertilizer	.383	.403	.376	.392	.385	.396
			ASH			
Ammonium Sulfate	7.04	6.95	7.04	7.17	6.57	7.04
Cyanamid	6.84	7.24	6.90	7.18	6.45	6.68
Complete	7.82	7.33	7.72	8.05	7.75	7.38
No Fertilizer	5.70	5.80	6.19	5.61	5.86	6.20
		$\frac{Ca + Mg}{K}$	RATIO			
Ammonium Sulfate	1.62	2.17	3.46	3.92	5.71	6.48
Cyanamid	1.93	2.69	4.14	4.75	5.97	6.31
Complete	1.99	2.50	4.06	4.91	6.44	7.05
No Fertilizer	1.64	2.01	3.08	3.94	4.63	5.87

place is shown in figures 1–6 for each of the chemical elements determined, and in figures 7–10 for each of the fertilizer treatments. Figure 11 shows curves representing the changes in the $\frac{Ca + Mg}{K}$ ratio for the different fertilizer treatments.

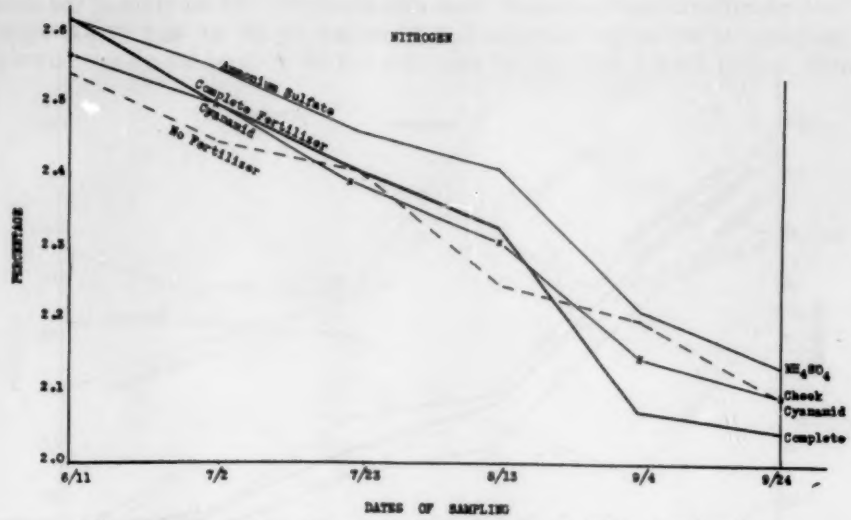

Fig. 1. Changes in nitrogen as influenced by fertilizer treatment.

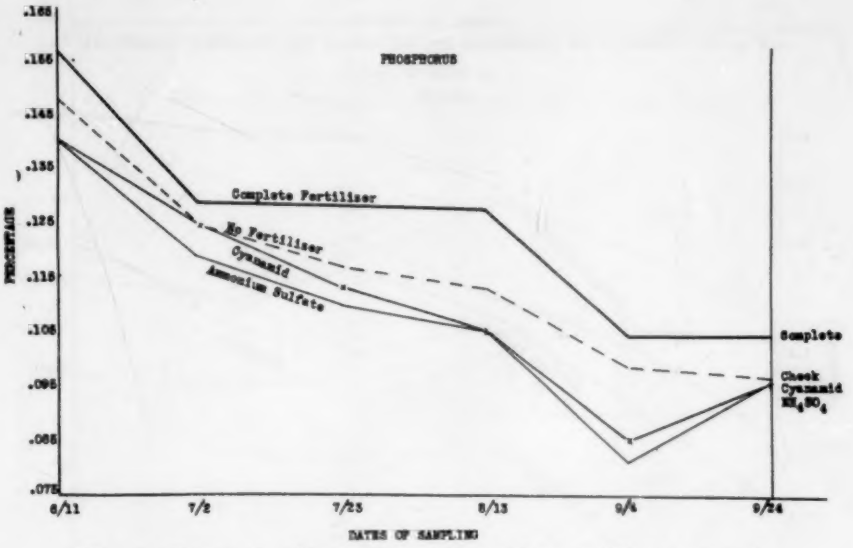

Fig. 2. Changes in phosphorus as influenced by fertilizer treatment.

Nitrogen, phosphorus, and potassium

The nitrogen, the phosphorus, and the potassium content of pecan leaves decreased more or less rapidly in all the fertilizer treatments from June 11 to September 24. However, two periods of more rapid decline were noted, the first occurring during the first two sampling periods, and the second between August 13 and September 4. The decrease was more pronounced

with phosphorus and potassium than with nitrogen. At the time of the third sampling of leaves, the nitrogen had decreased 31, 38, 31, and 27 per cent.;

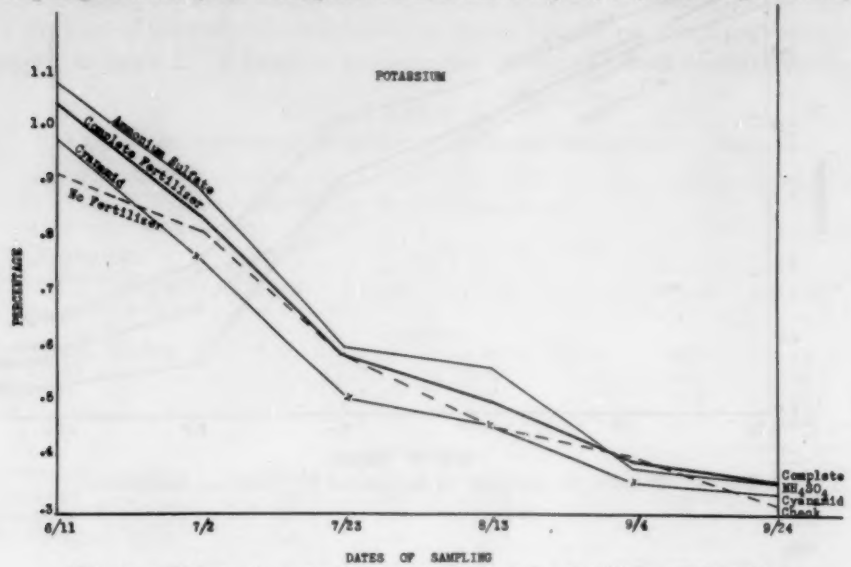

Fig. 3. Changes in potassium as influenced by fertilizer treatment.

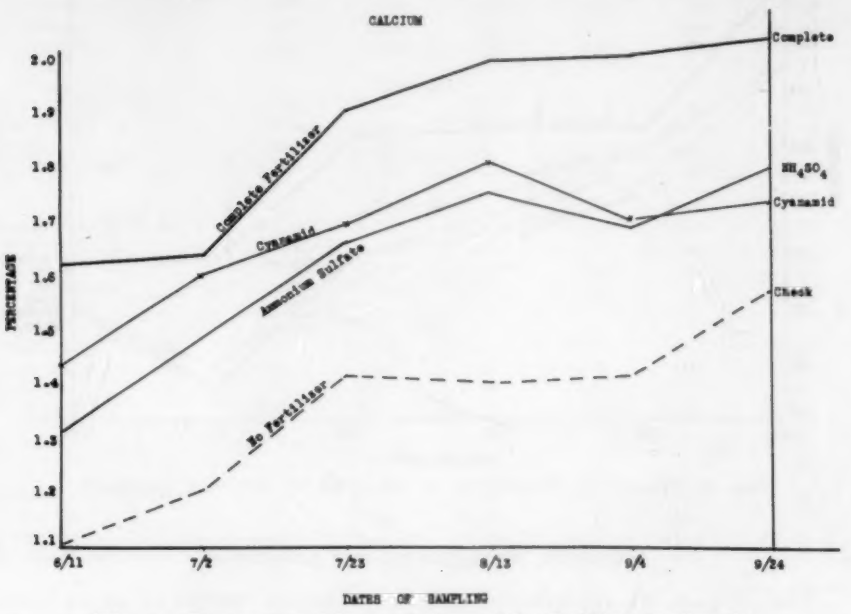

Fig. 4. Changes in calcium as influenced by fertilizer treatment.

phosphorus 67, 61, 56, and 59 per cent.; and potassium 65, 72, 67, and 58 per cent. of the total amount at the beginning of the period, respectively for ammonium sulfate, Cyanamid, complete-fertilizer, and no-fertilizer treat-

ments (figs. 7–10). In the leaves from the complete-fertilizer trees, phosphorus was found to drop 54 per cent. during the first 3-week period, then

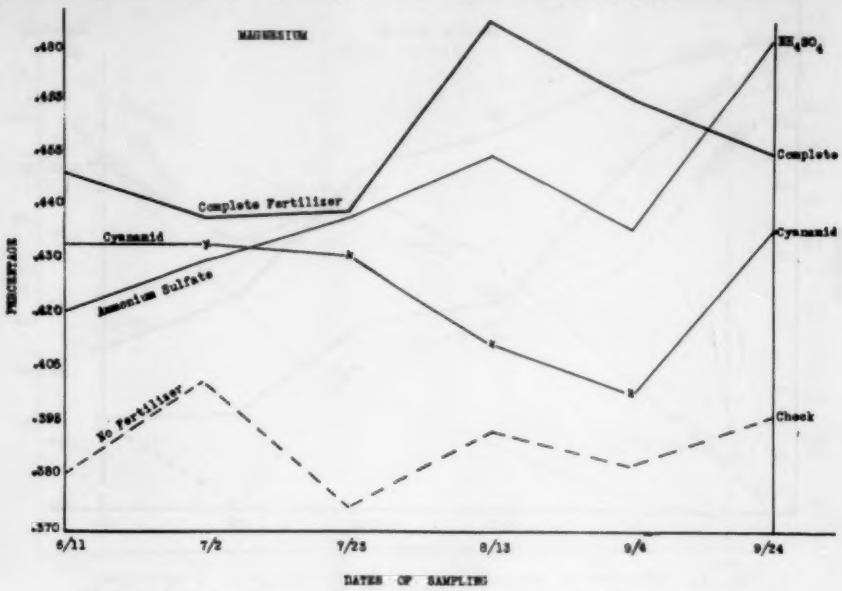

Fig. 5. Changes in magnesium as influenced by fertilizer treatment.

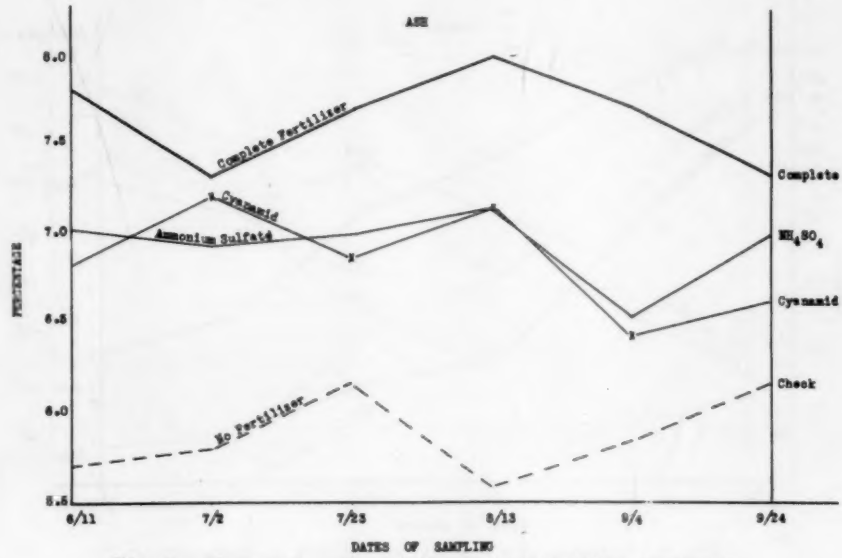

Fig. 6. Changes in ash as influenced by fertilizer treatment.

to remain constant for several weeks before the second marked decrease in late August. After September 4, the nitrogen and potassium content of the leaves continued to decrease more or less gradually in all treatments to

the end of the sampling period, while the phosphorus content tended to increase again.

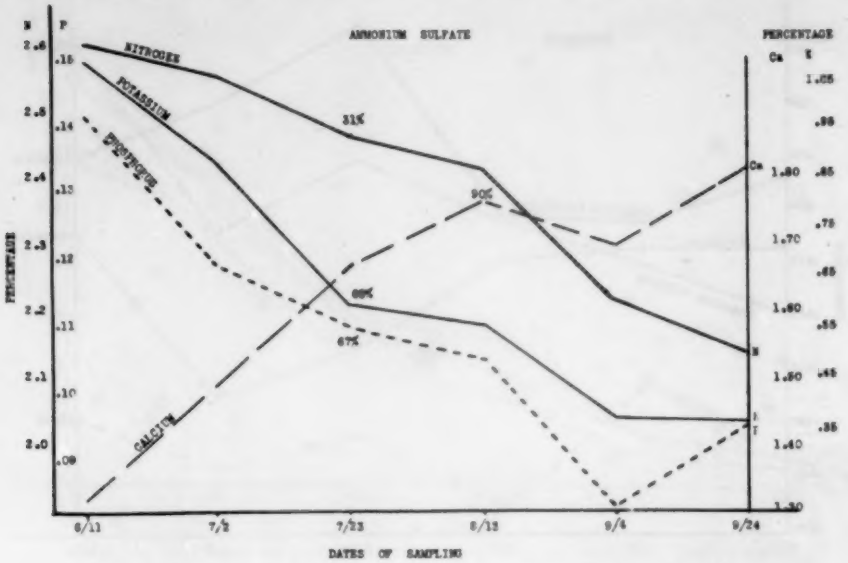

Fig. 7. Influence of Ammonium Sulfate on changes in composition.

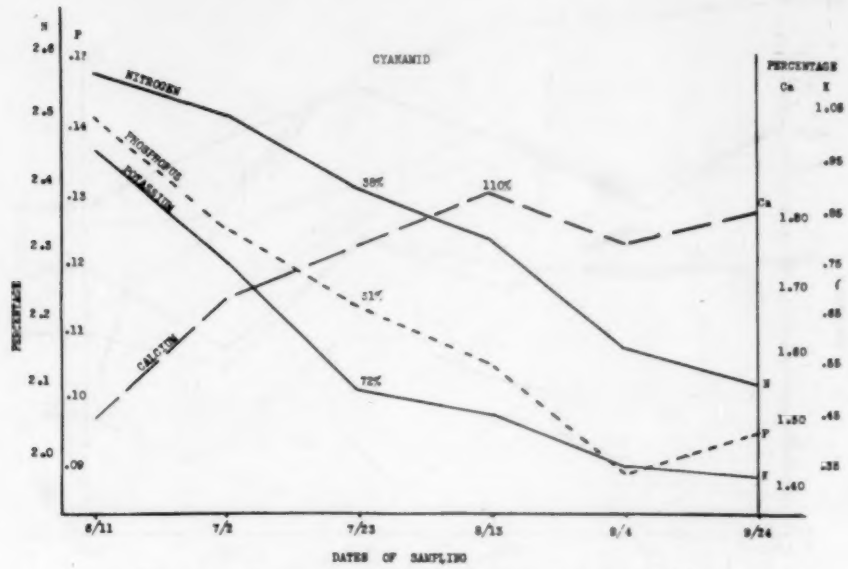

Fig. 8. Influence of Cyanamid on changes in composition.

Calcium, magnesium, and ash

The changes in the calcium and the magnesium content of the pecan leaves were opposite to those found for nitrogen, phosphorus, and potas-

sium. A very sharp rise in calcium occurred in the leaves from the trees in all the fertilizer blocks during the first four samplings. A general rise in the magnesium content of the leaves was observed for the entire period, but

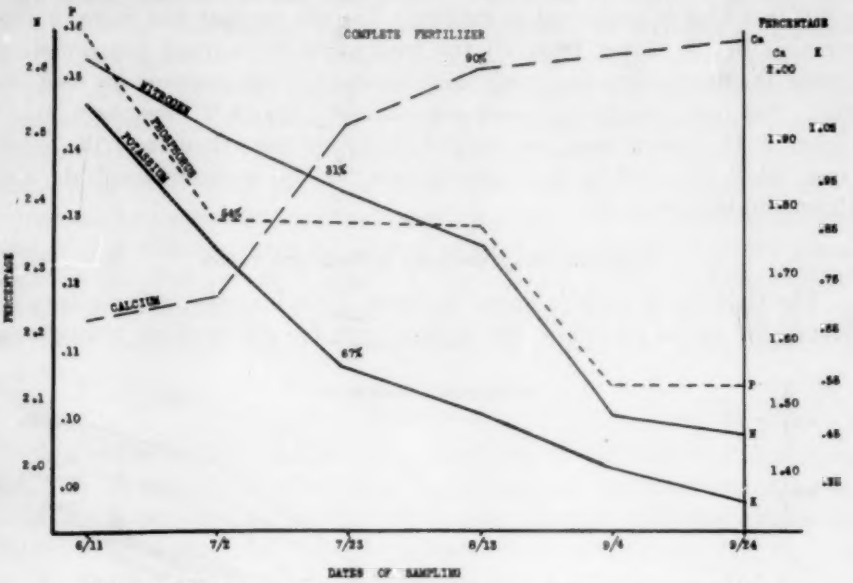

Fig. 9. Influence of Complete Fertilizer on changes in composition.

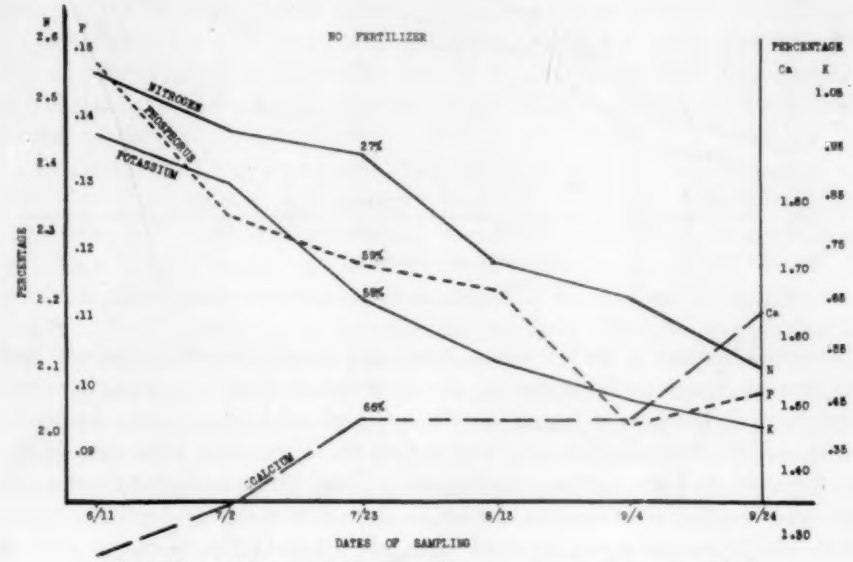

Fig. 10. Influence of No Fertilizer on changes in composition.

it was found to vary considerably between sampling dates and fertilizer treatments. After August 13 there was a drop in the calcium content in the two nitrogen-fertilizer blocks, while it continued to increase in the

complete-fertilizer and the no-fertilizer trees. Magnesium (fig. 5) was found to decrease more or less sharply in all treatments after August 13, but rose again after September 4 in all except the complete-fertilizer treatment, in which it continued to decline. The ash content was more or less constant in the leaves from all the treatments throughout the sampling period, the fluctuations occurring being similar to those observed for magnesium. Calcium, magnesium, and ash content (figs. 4–6) was each much higher in the leaves from the complete-fertilizer trees than from the check trees, while the content was intermediate for the ammonium-sulfate and Cyanamid-treated trees.

Calcium-magnesium: potassium ratio

The Ca + Mg/K ratio is shown in figure 11 to increase rather markedly during the entire period of nut development for all fertilizer treatments.

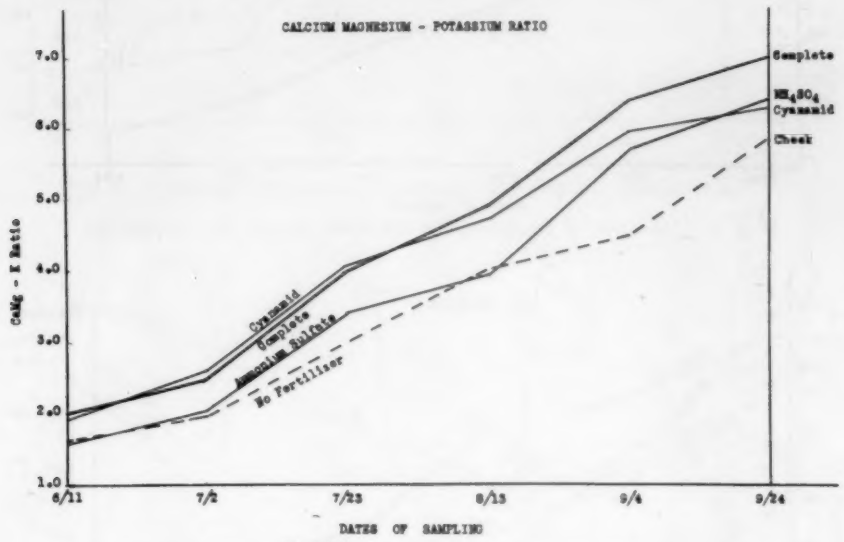

Fig. 11. Changes in $\frac{Ca + Mg}{K}$ ratio as influenced by fertilizer treatment.

The cation balance in the leaves was fairly low during the early summer, and differences between the different fertilizer treatments was quite narrow. However, as the season advanced, the ratio of calcium and magnesium to potassium became progressively higher and the differences were more striking between the four fertilizer treatments. The highest ratio and also rate of increase was found to exist in the leaves from the complete-fertilizer trees, while the lowest occurred in those from the no-fertilizer block.

Effect of fertilizer treatment

Fertilizer treatment was found to have a considerable influence on the level of nutrient elements in pecan leaves, and on the changes in these ele-

ments during the development period of the nut. The highest level of nutrients was found in the leaves from the complete-fertilizer block, and the lowest level for all the elements except phosphorus occurred in the non-fertilized trees. The lowest phosphorus level was found in the two nitrogen-fertilizer blocks. The highest percentage composition of nitrogen and potassium was maintained in the leaves from the ammonium sulfate treatment (figs. 1 and 3), while the highest phosphorus content was maintained in the trees receiving complete fertilizer (fig. 2). Potassium decreased more or less uniformly at a rapid rate in the leaves from all fertilizer treatments, the lowest level being found in those from the Cyanamid-treated trees. Cyanamid also seemed to have a depressing effect on the magnesium content (fig. 5). Fertilizer treatment seemed to have the least effect on the potassium content, but appeared to have a very striking influence on the calcium level and accumulation in pecan leaves. Similar trends occurred for all fertilizer treatments, the rate being much less pronounced in the non-fertilized trees.

Discussion

A number of investigators have noted that in the leaves of deciduous trees the content of chemical elements, particularly phosphorus and potassium, decreased rapidly during the summer months; and the content of these elements was much lower in mature leaves taken in the fall, than in young leaves taken earlier in the growing season. LILLELAND and BROWN (**14**) in studying the potassium content of leaves from nut and fruit trees, found that the percentage of this element decreased periodically from June 10 to October 1. The changes in potassium noted for walnut and filbert leaves showed trends remarkably similar to the results shown herein for pecans, though the changes reported were much less pronounced in the foliage of the walnut and filbert.

LILLELAND (**13**), working with fruit trees, states that the percentage of phosphorus, potassium, and nitrogen generally decreases with advancing age of the leaf, while the percentage of calcium, magnesium, sodium, and chlorine generally increases. In the case of various trees (**6, 15, 16, 17**) it has been shown that calcium increases steadily in the leaf to the time of leaf fall, while phosphorus and potassium decrease. This has been found in this investigation to be true of pecan leaves.

As it has been shown in previous papers (**10, 11**), the trees from which the pecan leaves were sampled supported an unusually heavy crop of nuts, which resulted in extremely poor filling. A shortage of phosphorus and potassium, and possibly of other chemical elements, or other factors during this period might account for an exaggerated condition reflected in the striking changes shown in the graphs. Accompanying this condition, leaf scorch appeared during July and progressed in severity through early August, a condition which was prevalent throughout the pecan area. It is possible that this scorch was caused by a deficiency of some chemical

element, or a multiple deficiency, at the specific time this condition developed, but in view of the fact that leaves from the same trees in 1943 had a slightly lower content of potassium (11) with no indication of scorch developing, it seems that some other factor is involved. It is believed that the scorch in 1945 may have been due to excessive rainfall (12.31 inches) in July following a hot dry spell when only 3.44 inches was recorded in this orchard in June. This correlates with observations on leaf scorch made by ALBEN (1) in Louisiana. No doubt excessive rainfall lowers the rate of photosynthesis of the leaf with resultant higher utilization of photosynthetic products held in reserve by the tree, and lowers the efficiency of the root system. The rapid deterioration and early defoliation of the leaves in early fall was no doubt due to depletion of mineral elements utilized in the production of the heavy crop of nuts; and failure of the nuts to fill properly was possibly due to the circumstance that much of the chemical reserves in the tree were utilized in the structural formation of the shell and shuck, together with unfavorable leaf conditions later in the season. It is believed that these conditions were not suitable for good nut development.

The first rapid decrease in the phosphorus and potassium content in pecan leaves during the first two sampling periods was possibly influenced by the degree of development of the leaf. Although the leaves sampled had reached normal size, active growing processes were no doubt taking place within the leaf whereby the actual dry weight of the leaf increased so rapidly that the percentage values of nitrogen and of some mineral constituents showed significant decreases. LILLELAND and BROWN (14) and MITCHELL (16) showed that potassium expressed on a unit basis of milligrams per leaf in the leaves of nut and fruit trees remained fairly constant or increased, whereas, when expressed on a percentage basis, a general decline was noted. This has also been brought out by PALLADIN (17), where it is shown that the percentage of phosphorus and potassium in the ash of beech leaves underwent a marked decrease during the course of the summer, while no corresponding decrease was apparent in the absolute amounts of these elements. This relative decrease in phosphorus and potassium might be influenced to some extent by the rapid accumulation of calcium as an ash constituent in the leaf during this period. Also the stress of the abnormally heavy crop load on the trees no doubt caused a heavy drain on the elements involved in the decreases. Another factor may have been deficiencies in these elements earlier in the season, especially in the trees of some of the treatments, such as those receiving nitrogen alone or the check.

It will be noted that pecan leaves have a lower content of potassium than many other fruit and nut trees, and from the data presented it would seem that a deficiency of this element was evident early in June. However, analyses over a period of years show that the initial contents of K preceding the crop of 1945 was normal for this period of sampling.

During the period of August 13 to September 4 there was a marked decrease of all the elements in all of the treatments except calcium in the com-

plete-fertilizer and check plots. This period marked the final stage in the structural development of the pecan nut and the hardening of the shell. It also marked the early filling stage of the kernels, the critical period when the greatest stress is felt in the nutrition of the tree and when the function of these elements becomes most important.

The very low level of calcium observed in the leaves from the non-fertilized block, as well as the less rapid accumulation of this element in the leaves from these trees, was reflected in the lower rate of decline in percentage of nitrogen, phosphorus, and potassium during the development period of the nut.

The ratio of calcium and magnesium to potassium showed a remarkable rate of increase during the entire period of sampling, and reached an abnormally high figure in the final sampling date, especially in the leaves from trees receiving complete fertilizer. The significance of the cation relationship has been emphasized by SHEAR, CRANE, and MYERS (**18**) in their work on boron deficiency and toxicity, showing this to be due to an unbalanced ratio between boron and the three major bases, calcium, magnesium, and potassium. It is highly probable that an unbalanced condition and definite deficiencies existed in the leaves reported herein. The rapid rise in the cation ratio might possibly account for the leaf scorch which occurred during mid-summer, and it is quite probable that the rapid deterioration of the leaves which took place during the later period was due to this relationship. The high ratio of calcium and magnesium to potassium might indicate that leaf scorch in pecan leaves is due to the deficiency of potassium which develops under the stress of a heavy crop of nuts.

While fertilizer treatment had a marked influence on the nutrient levels in the foliage of pecan trees, the fact that similar trends occurred in the seasonal changes of the several chemical elements shows that certain processes are taking place in the plant tissues which possibly cannot be altered by fertilizer treatment. However, the need for specific elements at definite periods in the development of the nut is shown by the levels and rates of changes in these elements as influenced by different treatments.

Summary

Pecan leaves from four differently treated fertilizer plots were sampled for chemical analyses at six samplings with 3-week intervals from June 10 to September 24, during the development and early filling period of the nut. Results are reported as percentages on dry-weight basis of nitrogen, phosphorus, potassium, calcium, magnesium, and ash, and are graphically presented for each of the elements determined and for the four fertilizer treatments. These treatments included complete fertilizer, Cyanamid only, ammonium sulfate only, and no fertilizer.

Very striking seasonal changes in the chemical composition of the leaves occurred for all fertilizer plots. In general, nitrogen, phosphorus, and

potassium were found to decrease more or less rapidly during the entire period, while calcium accumulated at an equally rapid rate.

Phosphorus and potassium were depleted in the leaf at a more rapid rate than nitrogen. Magnesium tended to increase slightly as the leaf matured, but content varied considerably between sampling dates and fertilizer treatments. The ash content remained more or less constant throughout the period; this may be because the depletion of phosphorus and potassium was offset by the accumulation of calcium, magnesium, and possibly other minerals, such as silicon, chlorides, and others.

The highest level of all elements was observed in the leaves from the trees receiving complete-fertilizer, while the lowest level was found in the unfertilized trees, except that phosphorus was lowest in the two nitrogen-fertilizer plots, and potassium was lowest in the Cyanamid treatment. The highest level of nitrogen and of potassium was found in the ammonium sulfate treatment, while that for phosphorus was found in the trees receiving the complete fertilizer.

The rate of decrease of nitrogen, phosphorus, and potassium, as well as the rate of accumulation of calcium, was much greater in the leaves from the fertilized trees than from those receiving no fertilizer. No doubt fertilizer treatment had a marked influence on the metabolism of nutrients in the leaf.

The $Ca + Mg/K$ ratio increased rapidly during the entire period of leaf sampling for all fertilizer treatments, the greatest rise occurring in the leaves of the trees receiving complete fertilizer, and the least in those receiving no fertilizer. A remarkably high ratio was reached in the leaves at the final sampling period. The rapid increase in this base relationship might account for the leaf scorch which developed during mid-summer, and the deterioration of the leaves in September.

Definite deficiencies in some of the elements developed in the leaves due to the stress of producing a heavy crop of nuts. This was especially evident for a deficiency in the potassium content of the leaves as shown by the $Ca + Mg/K$ ratio. Leaf scorch which developed on the leaves may have been caused by this or multiple deficiencies occurring at that time.

Two periods of rapid change in the concentrations of the chemical elements occurred in the leaves. The first period, including the first six weeks of sampling, marked a rapid decrease in nitrogen, phosphorus, and potassium, and a rapid accumulation of calcium. The second period of marked change occurred between August 13 and September 4, when all the elements except calcium were rapidly depleted. There was a period of a few weeks in mid-summer, possibly after active growing processes in the leaf tissue had ceased, during which the mineral content remained more or less constant.

The first period of rapid decrease in the chemical elements in the leaves was due either to the relationship of the content of elements in the growing leaf, or to the rapid utilization of these elements in growing processes under the stress of a heavy crop of nuts. The second period of rapid decline was

no doubt due to the utilization and translocation of these chemicals in the final stage of development and early filling of the nuts, during which the greatest stress in the nutrition of the trees is probably felt.

The striking chemical changes in the leaves from these trees were no doubt influenced to a marked degree by the fact that the trees were under the stress of producing a heavy crop of nuts, although fundamental deficiencies in some of the fertilizer treatments may partially account for these changes.

U. S. PECAN FIELD STATION
ALBANY, GEORGIA

LITERATURE CITED

1. ALBEN, A. O. Analyses of pecan leaves as an aid in fertility studies. Texas Pecan Grow. Assoc. Proc. **26**: 51–58. 1947.
2. ————————, and HAMMAR, H. E. Fertilizing the pecan. Texas Pecan Grow. Assoc. Proc. **19**: 48–54. 1939.
3. ————————, and ————————. Phosphoric acid content of Southwestern pecan soils and influence of phosphate fertilizers on pecan foliage. Soil Sci. Soc. Amer. Proc. **4**: 173–176. 1939.
4. ASSOCIATION OF OFFICIAL AGRICULTURAL CHEMISTS. Official and tentative methods of analysis. Ed. 4. Washington, D. C. 1935.
5. BROWN, D. C., ROBINSON, R. R., and BROWNING, G. M. Determination of small amounts of potassium. Indus. and Eng. Chem., Anal. Ed. **10**: 652–654. 1938.
6. CHANDLER, R. F., JR. The calcium content of the foliage of forest trees. Cornell Univ. Agr. Expt. Sta. Me. 228. 1939.
7. FINCH, A. H. Further notes on pecan filling and maturity. Amer. Soc. Hort. Sci. Proc. **32**: 24–28. 1934.
8. ————————, and VAN HORN, C. W. The physiology and control of pecan nut filling and maturity. Arizona Agr. Expt. Sta. Tech. Bul. **62**: 421–472. 1936.
9. HAMMAR, H. E., and HUNTER, J. H. Some physical and chemical changes in the composition of pecan nuts during kernel filling. Plant Physiol. **21** (4): 476–491. 1946.
10. HUNTER, J. H., and HAMMAR, H. E. The results of applying different fertilizers to the Moore variety of pecan over a ten-year period. Southeastern Pecan Grow. Assoc. Proc. **40**: 10–32. 1947.
11. ————————, and ————————. Nutritional deficiencies associated with poor filling of pecan nuts. Southeastern Pecan Grow. Assoc. Proc. **41**: 16–34. 1948.
12. LEWIS, R. D., and HUNTER, J. H. Changes in some mineral constituents of pecan nuts and their supporting shoots during development. Jour. Agr. Res. **68**: 299–306. 1944.
13. LILLELAND, OMUND. The present status of leaf analysis in relation to fruit tree nutrition. The Blue Anchor **23** (1): 4 p. 1946.

14. ————————, and Brown, J. B. The potassium nutrition of fruit trees II. Leaf analyses. Amer. Soc. Hort. Sci. Proc. **36**: 91–98. 1938.
15. McHargue, J. S., and Roy, W. R. Mineral and nitrogen content of the leaves of some forest trees at different times during the growing season. Bot. Gaz. **94**: 381–393. 1932.
16. Mitchell, H. L. Trends in nitrogen, phosphorus, potassium, and calcium content of the leaves of some forest trees during the growing season. Black Rock Forest Paper **1** (6): 30–44. 1936.
17. Palladin, Vladimir I. Palladin's Plant Physiology. Page 89. Ed. by B. E. Livingston. 2nd Amer. Ed. 360 pp. P. Blakiston's Son and Company. 1923.
18. Shear, C. B., Crane, H. L., and Myers, A. T. Nutrient-element balance: a fundamental concept in plant nutrition. Amer. Soc. Hort. Sci. Proc. **47**: 239–248. 1946.
19. Smith, C. L., and Thor, J. B. A physiological study of fruit development in the pecan (Hicoria pecan). Nat. Pecan Grow. Assoc. Bul. **32**: 46–49. 1933.
20. Thor, J. B., and Smith, C. L. A physiological study of seasonal changes in the composition of the pecan during fruit development. Jour. Agr. Res. **50**: 97–121. 1935.
21. ————————, and ————————. A physiological study of the prefilling period of fruit development in the pecan. Jour. Agr. Res. **58**: 905–910. 1939.
22. Woodroof, J. G., and Woodroof, N. C. The development of the pecan nut (Hicoria pecan) from flower to maturity. Jour. Agr. Res. **34** (11): 1049–1063. 1927.

EFFECTS OF PHOTOPERIOD AND TEMPERATURE ON GROWTH AND FLOWERING OF KENTUCKY BLUEGRASS[1]

MAURICE L. PETERSON AND W. E. LOOMIS

(WITH TWO FIGURES)

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Introduction

The responses of plants to varying photoperiod has received much attention since GARNER and ALLARD (**3, 4**) first showed that flowering and fruiting of many plants is controlled by the relative length of day and night. Numerous species have been classified as "short day" or "long day" on the basis of the day length required for maximum flowering and fruiting. Temperature also may influence the flowering responses of plants (**13**), and the two factors may show interaction (**11**).

The experiments with Kentucky bluegrass (*Poa pratensis* L.) reported here were conducted primarily for the purposes of studying the physiological response of this species to management practices which might influence forage and seed production (**9, 10**). The formation of flowers in May and June has placed this species in the "long-day" class. However, EVANS and WATKINS (**2**) found that bluegrass plants produced inflorescenses in the spring regardless of length of day, and none was reported in the fall under day lengths ranging from 8.5 to 18 hours. Since plants are dependent upon their leaves for both the stimulus of photoperiod (**5, 6**) and reserve accumulations, any cultural treatments such as close grazing or clipping of Kentucky bluegrass could influence subsequent seed production, either through induction or through subsequent development of flowers and fruits. An interaction of nitrogen fertilization with photoperiod also was found in studies reported by BORTHWICK (**1**).

Methods

Bluegrass sods five inches in diameter were cut from the field with a special cutter on November 30, 1944, trimmed to a depth of four inches, and potted in gallon crocks with a fertile field soil. Eighteen sods of each of three unnamed strains of grass, selected to show a range of vegetative types, were used to compare the effects of 11-, 15-, and 19-hour photoperiods at two temperatures upon growth and floral development in plants that had undergone normal fall temperatures and photoperiods. The 19-hour photoperiod was obtained by using artificial lights mornings and evenings. The 11- and 15-hour photoperiods were obtained by placing light-tight boxes over the remaining plants at appropriate times. Differences

[1] Journal paper no. J-1526 of the Iowa Agricultural Experiment Station, Project No. 1001.

in temperature readings inside and outside the boxes were determined to be insignificant. Greenhouse temperatures in the cool house during the experiment ranged from an average minimum of 55.8° to an average maximum of 65.4° F. The warm house ranged from 61.2° to 74.7° F. The number of shoots (innovations) per pot was counted at 25, 50 and 105 days after the start of the experiment as an index of rhizome development and vegetative proliferation, and flower panicles as a measure of flowering development.

When it became apparent that early fall conditions were involved in reproduction, two experiments were run to study the interaction of photoperiod and temperature in the induction and development of flower primordia of Kentucky bluegrass during the fall. In the first experiment, 24 sods of each of two strains were potted from the field on October 11, 1945. Half of the pots were brought at once into a warm greenhouse, kept at about 75° F., and placed under normal and 19-hour day lengths. The remaining plants were left out of doors under the same two photoperiods until December 7. The longer photoperiods were obtained by using artificial lights mornings and evenings. Five-hundred watt bulbs with reflectors were placed four feet apart and four feet above the plants.

Starting December 7, half of the plants from each of the four treatments were held in the warm greenhouse under 19-hour days and observed for the emergence of flower heads. The remaining plants were used to determine the amount of top growth and also the weight of roots and rhizomes present at the end of the induction period. The roots and rhizomes were then killed in hot, 80 per cent. alcohol, and used for a determination of available and reserve carbohydrates. Methods described by LOOMIS and SHULL (**8**) were used for sugars and polysaccharides.

A somewhat similar experiment was carried out in the fall and winter of 1947–48. In this experiment a tota' of 128 pots was used. Sods were potted from established bluegrass in August and allowed to grow under normal conditions out of doors until September 30. At that time the plants were divided into 16 comparable groups of 8 pots each. Treatments consisted of all possible combinations of the following: (1) Photoperiod: Normal fall (short) photoperiods; 18-hour photoperiods. (2) Temperature: Normal cool fall temperatures; warm (Ca. 75° F.) in greenhouse. (3) Fertilization: Unfertilized; 2 gm. $(NH_4)_2SO_4$ per one-gallon pot. (4) Clipping: Not clipped; clipped September 30 and October 24, at one-inch. All plants were placed in the warm greenhouse under 18-hour days on December 9 and observed for the emergence of flowering heads.

Experimental results

VEGETATIVE GROWTH

The 1944 experiments were set up with sods taken from the field on November 30, and grown in a warm or a cool greenhouse with photoperiods

of 11, 15 or 19 hours of light. Within three weeks after starting the experiment the average heights of leaves in the warm greenhouse were 16.1, 22.3 and 25.0 cm., respectively, for 11-, 15- and 19-hour light periods. For corresponding day lengths in the cool greenhouse the average heights were 9.2, 15.0 and 15.1 cm.

The number of shoots per pot was counted 25, 50 and 105 days after the start of the experiment. These data, summarized in table I, show

TABLE I

NUMBER OF SHOOTS (INNOVATIONS) PER POT OF THREE STRAINS OF KENTUCKY BLUEGRASS 25, 50, AND 105 DAYS AFTER THE PLANTS WERE PLACED UNDER THREE PHOTOPERIODS AT TWO TEMPERATURES

DAYS	STRAIN NO.	11 HOURS		15 HOURS		19 HOURS		AVERAGE
		WARM	COOL	WARM	COOL	WARM	COOL	
25	1	45.3	56.6	47.6	56.6	45.6	56.3	51.3
	2	59.6	67.3	71.9	49.0	61.3	56.9	61.0
	3	93.9	69.6	71.3	80.9	64.9	72.6	75.5
	Av.	66.3	64.5	63.6	62.2	57.3	61.9	62.6
50	1	67.6	94.9	72.3	76.6	68.6	77.6	76.3
	2	108.2	122.2	95.6	76.6	82.9	85.6	95.2
	3	138.2	119.2	91.9	103.9	92.6	89.9	106.0
	Av.	104.7	112.1	86.6	85.7	81.4	84.4	92.5
105	1	98.3	137.3	106.3	103.7	99.0	108.7	108.9
	2	143.3	173.6	117.3	119.3	106.0	124.7	130.7
	3	185.1	171.6	125.5	120.3	113.3	116.7	138.8
	Av.	142.2	160.8	116.4	114.4	106.1	116.7	126.1

slightly more shoots under 11-hour photoperiods after only 25 days, although the differences were not statistically significant (**12**), table II. After 50 days there was an average of 23.3 more shoots per pot under 11-hour days than under 19-hour days at the warm temperature. Results with cool temperatures or with 15-hour photoperiods were not significantly different. Differences between short and long photoperiods were significant beyond the five per cent. level. Strain differences were highly significant in all counts.

Differences in shoot numbers continued to widen during the next 55 days, showing maximum average differences under cool temperatures. The 11-hour photoperiods under cool temperatures had an average of 160.8 shoots per pot, an increase of 48.7 from the previous count and 2½ times the early number. By comparison, the 19-hour photoperiods had 116.7 shoots or an increase of 32.3 since the 50-day count. It is a common observation that bluegrass stands tend to thicken in the autumn, and these data suggest that this thickening is in part a photoperiod response.

The top growth from the 54 pots of the main experiment was harvested, dried, and weighed on February 15, and again one month later. Yields

TABLE II

ANALYSIS OF VARIANCE OF NUMBER OF SHOOTS OF KENTUCKY BLUEGRASS PER POT WHEN GROWN UNDER DIFFERENT PHOTOPERIODS AND TEMPERATURES AND COUNTED 25, 50 AND 105 DAYS AFTER STARTING THE EXPERIMENT

Source of variation	Df.	Mean squares		
		25 days	50 days	105 days
Temperatures	1	3.13	143.41	1102.51
Reps. within temperatures	4	124.30	144.07	444.63
Photoperiods	2	151.13	3493.13*	8826.90†
Photoperiods × temperatures	2	59.24	73.24	483.14
Error (a)	8	215.49	503.13	815.80
Strains	2	2676.08†	4048.46†	4317.46†
Strains × temperatures	2	347.63	363.36	689.36
Strains × photoperiods	4	68.02	427.41	1098.69
Strains × temp. × photo.	4	423.58	472.68	308.29
Error (b)	24	227.29	370.25	625.49
Total	53			

* F value exceeds 5 per cent. level of significance.
† F value exceeds 1 per cent. level of significance.

from the earlier harvest were considerably larger under the longer photoperiods, as shown in table III. The weight of tops was consistently greater under warm temperatures. Analysis of variance of these data show highly significant differences between photoperiods and temperatures. Strain differences also were highly significant, with strain 3 yielding 16 per cent. more than strain 1. The significant interaction of strain × temperature resulted from much larger strain differences under the warm temperatures.

TABLE III

AVERAGE OVEN-DRY WEIGHT OF HERBAGE, IN GRAMS PER POT, PRODUCED BY THREE STRAINS OF KENTUCKY BLUEGRASS GROWN UNDER DIFFERENT PHOTOPERIODS AND TEMPERATURES

Strain	11 hours		15 hours		19 hours	
	February 15	March 15	February 15	March 15	February 15	March 15
	Warm temperature					
1	4.80	4.23	7.37	5.30	7.31	4.60
2	5.36	5.50	8.14	5.60	7.14	4.80
3	6.83	6.60	8.52	6.43	9.13	5.73
Average	5.66	5.44	8.01	5.78	7.86	5.04
Ratio Feb./Mar.	1.04		1.39		1.56	
	Cool temperature					
1	3.30	4.85	6.19	4.80	6.75	4.43
2	3.14	5.00	5.94	5.30	6.36	4.93
3	3.57	5.60	6.65	5.70	6.82	5.27
Average	3.33	5.14	6.26	5.27	6.64	4.88
Ratio Feb./Mar.	0.65		1.19		1.36	

Yields obtained March 15, one month after the first clipping, are believed to have reflected differences in the amount of carbohydrates stored during the previous growth period. There was an indication that storage was greater under the shorter photoperiods, as illustrated particularly by plants under cool temperatures, which produced more on the March clipping under 11-hour photoperiods, while under 19-hour days yields in March were lower than in February.

Carbohydrate reserves

Half of the 1945 plants were used for green weights of tops and roots and chemical samples of roots plus rhizomes at the time of ending the induction treatments on December 7. These plants were brought from the field on October 11, and were held under normal or long days, in a warm greenhouse, or outside with temperatures well below freezing at the end of the period. The effects of these treatments upon growth and storage of reserves are shown in table IV.

Determinations were made of reducing sugars, sucrose, total sugars and water soluble polysaccharides, mainly dextrins. It was assumed that starch was not present in either roots or rhizomes since it was not demonstrable with iodine and since samples treated overnight with fresh saliva at about 35° C. gave the same results as water extractions alone. Clearing of the polysaccharide extract with neutral lead acetate was found unnecessary.

In these experiments, analyses were made of the entire underground parts (roots plus rhizomes). Previous work had shown that the total carbohydrate content of roots was only about one-fourth that of the rhizomes. Sucrose was an important carbohydrate reserve (table IV) and showed the greatest variation between treatments, ranging from 259 to 1291 mg. per 100 grams. Reducing sugars accounted for about one-fifth to one-third of the total sugars. The water soluble polysaccharides were less affected than total sugars, being about the same for the outdoor fall photoperiods and temperatures and the 19-hour photoperiods and greenhouse temperatures.

Top growth was greatest under greenhouse temperatures and long photoperiods and least under outside temperatures and normal (short) photoperiods. Also the ratios of roots to tops were largest under the latter condition and smallest under the long, warm days. Plants with the highest ratios (i.e., little top growth compared with roots) showed the greater carbohydrate storage. The three pots of strain 1, which combined had only 3.3 grams of top growth (fresh weight), supported 63 grams of roots plus rhizomes and enabled them to pile up 3257 mg. total carbohydrates per 100 grams of fresh tissue. Strain 2 had more root and top growth but was apparently less efficient in storing carbohydrate reserves.

Storage under the 19-hour photoperiods at outside temperatures was considerably less for strain 1 and slightly less for strain 2. In comparing photoperiod effects with temperature effects it is apparent that temperature was more important for carbohydrate storage as well as for top growth.

TABLE IV

FRESH WEIGHT OF ABOVE AND BELOW GROUND PARTS, AND CARBOHYDRATE CONTENT EXPRESSED AS MILLIGRAMS PER 100 GRAMS FRESH TISSUE OF ROOTS PLUS RHIZOMES OF KENTUCKY BLUEGRASS PLANTS HELD UNDER DIFFERENT PHOTOPERIODS AND TEMPERATURES FROM OCTOBER 11 TO DECEMBER 7

MEASUREMENT	NORMAL FALL PHOTOPERIOD				19-HOUR PHOTOPERIOD			
	OUTSIDE		GREENHOUSE		OUTSIDE		GREENHOUSE	
	STRAIN 1	STRAIN 2	STRAIN 1	STRAIN 2	STRAIN 1	STRAIN 2	STRAIN 1	STRAIN 2
Grams roots + rhizomes (3 pots)	63.0	76.0	90.0	84.0	73.0	81.0	61.0	81.0
Grams tops (3 pots)	3.3	3.8	17.6	21.3	5.3	7.4	22.9	19.9
Root/top ratio	18.9	19.8	5.1	4.0	13.9	11.0	2.7	4.1
Reducing sugars	459	267	174	107	334	270	183	111
Sucrose	1291	1041	327	399	934	766	515	259
Total sugars	1818	1363	518	527	1317	1076	725	384
Water soluble polysaccharides	1439	903	895	776	1396	1068	1304	788
Total carbohydrates	3257	2266	1413	1303	2713	2144	2029	1172

The total carbohydrates bear a close relationship to the root/top ratio except that strain 1 under 19-hour photoperiods and greenhouse temperatures appears high in comparison with the other treatments.

Floral induction

The production of an inflorescence involves the differentiation of a flower primordium (7) and also the subsequent growth of the floral parts and culm. Since we made no histological observations for the appearance of flower primordia, these two phases of development cannot be completely separated with the data available. The indications, however, are reasonably distinct. Both short days and cool temperatures appear to be essential for the induction of the flowering condition in Kentucky bluegrass (table V). In this experiment the plants which had been under the differ-

TABLE V

NUMBER OF FLOWERING HEADS PER POT OF KENTUCKY BLUEGRASS AFTER INDUCTION UNDER DIFFERENT PHOTOPERIODS AND TEMPERATURES FROM OCTOBER 11 TO DECEMBER 7, 1945, AND DEVELOPING UNDER 19-HOUR PHOTOPERIODS AND WARM GREENHOUSE TEMPERATURES AFTER DECEMBER 7

Strain no.	Replication	Normal fall photoperiod		19-hour photoperiod	
		Outside	Greenhouse	Outside	Greenhouse
1	1	3	0	0	0
	2	3	0	0	0
	3	1	0	0	0
	Total	7	0	0	0
2	1	6	0	0	0
	2	0	0	2	0
	3	1	0	0	0
	Total	7	0	2	0
Total both strains		14	0	2	0

ent conditions during the fall induction period were moved to a warm greenhouse (about 75° F.) and 19-hour photoperiods. Flowering heads appeared on plants which had been kept under normal fall conditions within a few weeks after moving. Temperatures cooler than those in the greenhouse seem to be necessary for the induction process as no heads appeared on plants kept under the short days in the greenhouse. In the pots from the 19-hour photoperiod only one plant produced two flower heads. This plant was held under the normal cool to freezing autumn temperatures.

A similar but more comprehensive experiment run in the fall and winter of 1947–48, confirmed the results of the earlier experiments (table VI, figs. 1 and 2). In this study nitrogen fertilization (2 gm. $(NH_4)_2SO_4$ per pot) and clipping treatments were superimposed upon the photoperiod and temperature experiments. These treatments would be expected to act upon flower growth rather than induction. The fertilized plants made a good

leaf growth under the short photoperiods. The foliage was dark green, prostrate, and exceedingly dense. Unfertilized plants made little fall growth and were pale green in color. The fertilized pots later produced five times more flowering heads, indicating that growth may be seriously limiting for flowering and seed production in Kentucky bluegrass.

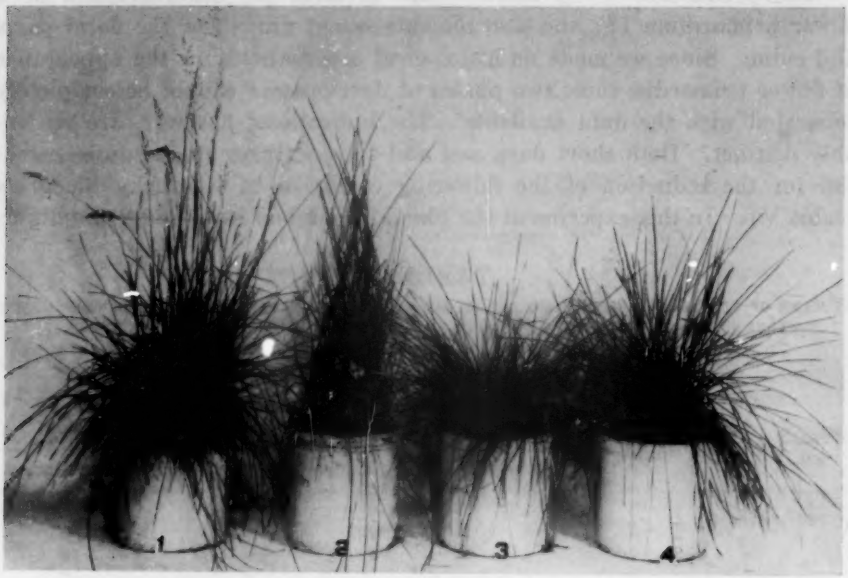

FIG. 1. Effect of temperature and photoperiod on floral induction and development in Kentucky bluegrass. These plants received the following treatments during the period September 30 to December 9, 1947; (1) short photoperiod and cool temperature; (2) 18-hour photoperiod and cool temperature; (3) normal photoperiod and warm temperature; and (4) 18-hour photoperiod and warm temperature. Only plants from treatment (1) produced flowers. All plants were placed under 18-hour photoperiods and warm temperatures on December 9. Photograph taken January 16, 1948.

The clipped but fertilized pots produced only nine heads compared with 107 heads on unclipped pots. Clipping treatments were moderately severe with two clippings on September 30 and October 24, and about one-inch of the stubble left at each clipping. Clipping also would be expected to affect the growth of flowers rather than their induction, and it is significant that the effects of both fertilization and clipping were quantitative rather than qualitative. We consider it a reasonable assumption, and one to be studied in further work, that the clipped or unfertilized plants produced many flower primordia which were unable to develop into inflorescences.

In this experiment, as in the previous one, a very few heads (2 on 16 pots) were produced on plants which were kept out-of-doors under the long photoperiod, but plants from both the long-day and the high temperature treatments were essentially non-flowering.

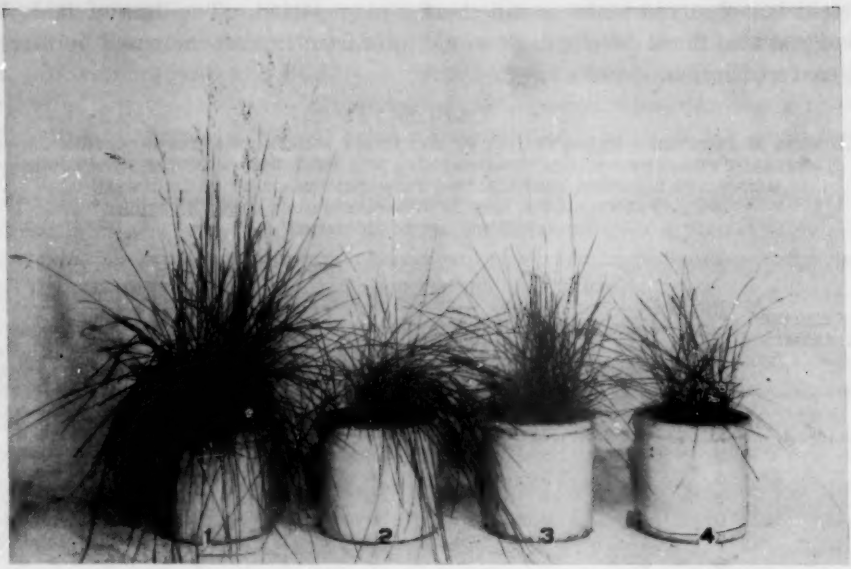

FIG. 2. Effect of clipping and fertilization on floral induction and development in Kentucky bluegrass. These plants had undergone the normal fall temperatures and photoperiods favorable for the induction of flowers from September 30 to December 9. They also received the following clipping and fertilizer treatments: (1) fertilized and unclipped; (2) fertilized and clipped; (3) unfertilized and unclipped; and (4) unfertilized and clipped. The number of inflorescences developing was proportional to the vigor of the plants. Plants were placed under 18-hour photoperiods and greenhouse temperatures on December 9. Photograph taken Jan. 16, 1948.

FLORAL DEVELOPMENT

To determine the temperature and day lengths favorable for the emergences of flowering heads after the plants had undergone the short fall photoperiods and cool temperatures favorable for induction, plants which had developed under normal fall conditions until November 30, were placed under 11-, 15- and 19-hour photoperiods in both warm and cool greenhouses. The number of heads emerging between December 22, when the first heads appeared, and March 1 are shown in table VII.

Heads developed under all conditions of temperature and photoperiod; under the cool temperatures, however, more than three times as many heads were produced as under the warmer temperatures. Only four heads were produced under the 11-hour photoperiods compared with 44 and 40 heads under the 15- and 19-hour photoperiods. The response of the plants to the long photoperiods appeared to be similar under both temperatures. It is probable that even the 15-hour photoperiod is longer than is required for satisfactory emergence of flower heads. As an average, bluegrass heads emerge about May 15 at Ames. The day length from sunrise to sunset at that date is 14 hours and 33 minutes, and early flower develop-

ment has occurred under a still shorter photoperiod. The data of table V suggest that floral development would have been farther increased by nitrogen fertilization.

TABLE VI

NUMBER OF FLOWERING HEADS PER POT OF KENTUCKY BLUEGRASS AFTER INDUCTION UNDER DIFFERENT PHOTOPERIODS AND TEMPERATURES AND WITH DIFFERENT CLIPPING TREATMENTS AND NITROGEN FERTILIZATION FROM SEPTEMBER 30 TO DECEMBER 9, 1947. FLOWERS DEVELOPED IN THE GREENHOUSE UNDER 18-HOUR PHOTOPERIODS AFTER DECEMBER 9

CLIPPING TREATMENT	REPLICATION	NORMAL FALL PHOTOPERIOD				18-HOUR PHOTOPERIOD			
		OUTSIDE		GREENHOUSE		OUTSIDE		GREENHOUSE	
		0.4 GM. N	NONE	0.4 GM. N	NONE	0.4 GM. N	NONE	0.4 GM. N	NONE
NOT CLIPPED	1	6	2	0	0	0	0	0	0
	2	14	0	0	0	0	0	0	0
	3	9	5	0	0	0	0	0	0
	4	13	3	0	0	0	0	0	0
	5	13	0	0	0	0	0	0	0
	6	4	2	0	0	0	0	0	0
	7	17	5	0	0	1	0	0	0
	8	31	5	0	0	1	0	0	0
	TOTAL	107	22	0	0	2	0	0	0
CLIPPED SEPT. 30 AND OCT. 24	1	0	0	0	0	0	0	0	0
	2	0	0	0	0	0	0	0	0
	3	2	0	0	0	0	0	0	0
	4	2	3	0	0	0	0	0	0
	5	1	0	0	0	0	0	0	0
	6	1	0	0	0	0	0	0	0
	7	2	0	0	0	0	0	0	0
	8	1	0	0	0	0	0	0	0
	TOTAL	9	3	0	0	0	0	0	0

TABLE VII

THE INFLUENCE OF PHOTOPERIOD AND TEMPERATURE UPON THE DEVELOPMENT OF FLOWERS OF KENTUCKY BLUEGRASS PLANTS WHICH HAD UNDERGONE THE NORMAL FALL TEMPERATURES AND PHOTOPERIOD FAVORABLE TO THE INDUCTION OF FLOWER PRIMORDIA

AVERAGE TEMPERATURE RANGE	11 HOURS	15 HOURS	19 HOURS	TOTAL
61.2°–74.7° F.	3	11	7	21
55.8°–65.4° F.	1	33	33	67
Total	4	44	40	88

Discussion

Kentucky bluegrass shows in the fall what appears to be a typical short-day response in a prostrate habit of growth, rapid accumulation of reserves and proliferation of new rhizomes and shoots. Shoot development seemed to be nearly independent of temperature over the range studied (table I), but carbohydrated accumulation was more closely related to temperature

than to photoperiod. In the 1945 experiments (table IV) the average accumulation of reserves was slightly increased by the short photoperiod at low temperatures and slightly decreased in the greenhouse. The overall effect of low temperatures, regardless of photoperiod, however, was a near doubling of the percentage of total carbohydrates in spite of the small top growth produced with these treatments.

Our experiments show that the flower buds of Kentucky bluegrass differentiate in the fall, although the possibility of further differentiation in the very early spring has not been eliminated. The induction appears to be the combined effect of low temperature and short photoperiod, and essentially no flowering occurred, two flower stalks in 64 pots, when only one of these factors was present. None was produced when neither factor was present. The normal day length (sunrise to sunset) at Ames on October 11 is 11 hours and 17 minutes. By November 1, the length of day has decreased to 10 hours and 22 minutes and by December 7, when the "induction" phase of our experiments was concluded, the day length is 9 hours and 15 minutes. By the latter date the plants and soil were completely frozen and physiological processes in the plant essentially at a standstill. Little if any visible signs of growth appear before March 15 at Ames in the average spring. At this date the day length is 11 hours and 53 minutes. By April 1 this has lengthened to 12 hours and 42 minutes. The minimum photoperiod necessary for floral induction has not been determined in these experiments. Essentially, however, the fall photoperiod and temperature conditions are duplicated for a short time in early spring, and floral induction may be possible at this time provided the necessary leaf area has developed. Our experiments indicate that fall induction is normal for this species.

After the short photoperiods and cool temperatures, longer photoperiods at moderately low temperatures favor the growth and final emergence of the heads (table VII) and the classification of the plant as "long day" or "short day" will depend upon whether the induction or development period is used. The dependence of vigor of flowering upon fall cultural treatments is indicated in table VI. Only the fertilized, unclipped pots produced normal numbers of flower heads after they were brought into the greenhouse. In earlier work one of us showed (9) that fall deferment (no clipping or pasturing) did not increase herbage yields the following spring. No counts were made, but the present results suggest that the extra fall growth may have increased flower bud development and stimulated flowering in the following spring at the expense of vegetative growth.

Summary

Studies were conducted with Kentucky bluegrass (*Poa pratensis*) through the fall and winter seasons of 1944, 1945 and 1947 to determine the response of this species to different photoperiods and temperatures,

particularly during the fall. The following appear to be the primary responses.

1. The devolopment of shoots (innovations) was increased by short days (11 hour) and seemed to be influenced little by temperature differences. Prostrate growth habit was also a short-day effect.

2. Carbohydrate accumulation was more closely related to cool temperatures than to short photoperiods, but both factors combined produced the greatest carbohydrate storage.

3. The induction of the flowering condition occurred during the fall under normal cool temperatures and short days, and appeared to be the combined effect of low temperature and short photoperiod. No flowers were produced on plants which had been held at warm temperatures during the induction period, and long photoperiods had essentially the effect of preventing flowering, regardless of temperature.

4. Nitrogen fertilization in early fall stimulated vegetative growth and tillering, and increased the number of flowers subsequently produced to about five times more than unfertilized plants. Clipping during the fall (September 30 and October 24) reduced the number of flowers to about 1/10 the number produced on unclipped, fertilized plants and essentially prevented flowering of unfertilized plants. These responses are considered to be due to reduced flower growth rather than to differences in induction or differentiation.

5. The development of flowers after the grass had undergone the cool temperatures and short photoperiods was favored by longer photoperiods and moderately low temperatures. Therefore, the classification of bluegrass as a "short day" or "long day" plant will depend upon whether the induction or the development period is being considered.

AGRONOMY AND BOTANY AND PLANT PATHOLOGY SECTIONS
IOWA STATE COLLEGE
AMES IOWA

LITERATURE CITED

1. BORTHWICK, H. A. Day length and flowering. Science in Farming. Yearbook of Agriculture. pp. 273–283. 1943–1947.
2. EVANS, M. W., and WATKINS, J. M. The growth of Kentucky bluegrass and of Canada bluegrass in late spring and in autumn as affected by the length of day. Jour. Amer. Soc. Agron. **31**: 767–774. 1939.
3. GARNER, W. W., and ALLARD, H. A. Effect of the relative length of day and night and other factors of the environment on growth and reproduction in plants. Jour. Agr. Res. **18**: 553–606. 1920.
4. ————. Further studies in photoperiodism, the response of the plant to relative length of day and night. Jour. Agr. Res. **23**: 871–920. 1923.

5. HAMNER, KARL C. Interrelation of light and darkness in photoperiodic induction. Bot. Gaz. **101**: 658–687. 1940.
6. ———— and BONNER, JAMES. Photoperiodism in relation to hormones as factors in floral initiation and development. Bot. Gaz. **100**: 388–431. 1938.
7. LOOMIS, W. E. Growth-differentiation balance vs. carbohydrate-nitrogen ratio. Proc. Amer. Soc. Hort. Sci. **29**: 240–245. 1932.
8. ———— and SHULL, C. A. Methods in Plant Physiology. 472 pp. McGraw-Hill Book Co., New York. 1937.
9. PETERSON, MAURICE L. Physiological response of Kentucky bluegrass to different management treatments. Thesis, Iowa State College. 1946.
10. ————. Effect of method of grazing unimproved Kentucky bluegrass on beef production, botanical composition, and herbage yields. Jour. Amer. Soc. Agron. **39**: 412–422. 1947.
11. ROBERTS, R. H., and STRUCKMEYER, B. E. The effect of temperature and other environmental factors upon the photoperiodic responses of some of the higher plants. Jour. Agr. Res. **56**: 633–677. 1938.
12. SNEDECOR, GEORGE W. Statistical Methods. 4th ed., 485 pp. Collegiate Press, Inc., Ames, Iowa. 1946.
13. THOMPSON, H. C. Further studies on effect of temperature on initiation of flowering in celery. Proc. Amer. Soc. Hort. Sci. **45**: 425–430. 1944.

THE USE OF GROWTH-REGULATING CHEMICALS TO INDUCE PARTHENOCARPIC FRUIT IN THE CALIMYRNA FIG

JULIAN C. CRANE AND RENE BLONDEAU

(WITH TWO FIGURES)

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Introduction

Certain varieties of many species of plants develop their fruits parthenocarpically or without fertilization. The apple and pear occasionally produce parthenocarpic fruits but generally under special nutritive conditions (**10**). In some varieties of grapes, figs, bananas, and oranges, parthenocarpy is of great economic importance (**4, 14**). Because of this naturally occurring phenomenon, attention has been directed toward inducing parthenocarpy in other fruits and vegetables by the application of hormones or various synthetic growth-regulating substances.

Early investigations by GUSTAFSON (**9**) on chemically-induced parthenocarpy stimulated research in this field and, at the present time, it is possible to produce parthenocarpically several different vegetables, as well as fruits of some ornamental plants. Similar attempts to increase fruit set or to induce seedlessness in some of our more important tree crops, such as the apple, orange, and grapefruit (**1, 8, 11, 15**) have failed entirely. A review of literature has revealed that all attempts to promote parthenocarpic fruit development in a temperate zone commercial tree fruit, where it did not already occur naturally, have been unsuccessful. This paper presents the results of preliminary experiments that were successful in chemically inducing parthenocarpic development of fruit of the Calimyrna fig.

Of the four most important fig varieties produced in California, the Mission, Adriatic, and Kadota, which are considered to be of the common type, are characterized by being completely parthenocarpic. On the other hand, the Calimyrna, the leading drying variety in the state, is of the Smyrna type, and exhibits the typical characteristics for this group by being almost completely non-parthenocarpic. The syconia of the Smyrna type mature only after the flowers have been pollinated and the ovules fertilized.

Pollination of the Calimyrna variety is accomplished by the fig-wasp, *Blastophaga psenes* L., which carries pollen from the male or caprifig to the stigmas of the Calimyrna, a process termed caprification. Thus in the production of Calimyrna figs the use of caprifigs and fig-wasps is indispensible. However, accompanying commercial caprification are several problems of vital concern to the grower. He must either maintain caprifig

trees in his orchard, which occupy valuable land area, or purchase caprifigs from other growers. In either case there is the risk that the caprifigs and the Calimyrna figs will not reach the proper stage of development at the same time. Placing the caprifigs in the orchard is laborious, expensive, and is, of necessity, based largely on experience rather than on a standardized procedure. Over- or under-caprification must be avoided, an optimum that is very difficult to achieve. The former favors the incidence of internal rot or endosepsis, a disease spread by the wasps, while the latter reduces the quality and quantity of yield. For a complete discussion of the process of caprification and its problems the papers of CALDIS (**2**), CONDIT (**5**), and SMITH and HANSEN (**12, 13**) are suggested.

The entire operation of caprification is costly, and to say the least, haphazardous. It is plainly evident that the fig grower would be interested in new techniques which have as their objective elimination of caprification from the list of cultural operations associated with the production of Calimyrna figs. With this in mind, investigations were initiated to determine the possibility of promoting parthenocarpic fruit development by the use of certain synthetic growth-regulating chemicals.

Materials and methods

Three synthetic growth-regulating chemicals were used: 2,4-dichlorophenoxyacetic acid (2,4-D), β-naphthoxyacetic acid (NOA), and γ-(indole-3)-n-butyric acid (IBA). All three were applied as water suspensions or solutions, and, in addition, the latter two were applied in oil emulsified in water. For the water-based formulations, the 2,4-D and NOA were dissolved in a minimum amount of ethanol, which was then diluted to give clear solutions. The IBA was prepared according to the method given by WITHROW and HOWLETT (**16**), using ethanol and gum arabic. A preparation containing both NOA and IBA was made by combining the stock solutions in the desired proportions. The oil-based formulations were prepared by dissolving the NOA and IBA in a minimum amount of normal butanol. This solution was then added to a light emulsive oil, the normal butanol serving as a mutual solvent. The oil stocks were then vigorously hand shaken with the required amount of water to give emulsions of the desired concentration of active material. Here also, a preparation containing both NOA and IBA was made by combining the stock solutions in the desired proportions.

Two methods of application were employed. The first consisted of injecting with hypodermic syringes the preparations (exclusive of the oil emulsions) into the small figs through their ostioles. The amount used varied with the size of the fig, but in every case the fig cavity was filled. The second method consisted of spraying the entire branch. With the water-based materials a nasal atomizer, connected to an air compressor, was used to give complete coverage and minimize spray drift. A paint gun,

operated at five lbs. pressure, was used in applying the oil emulsions to give a light deposit approximately equivalent to 25 gallons per acre.

The following table summarizes the materials, concentrations, and methods of application that were employed.

Material	*Concentration*	*Water-based*		*Oil-based*
2,4-D	10 and 5 p.p.m.	Injected	Sprayed	.....
NOA	100 and 50 p.p.m.	"	"	Sprayed
IBA	2670 and 1500 p.p.m.	"	"	Sprayed
NOA + IBA	100 + 2670 p.p.m.	"	"	.....
NOA + IBA	50 + 1500 p.p.m.	"	"	.....
NOA + IBA	25 + 750 p.p.m.	.....	.....	Sprayed

The above materials and concentrations were selected after a general survey of the literature was made on chemically induced parthenocarpy. Treatments were made on three different dates, May 24, June 5, and June 12, 1947, using a different set of branches on each date. These dates approximated the beginning, the middle, and the end of the normal caprification period. Paper bags containing caprifigs were placed in the orchard on May 29 and removed on June 20. The branches used were selected for uniformity of size and number of small figs, and all treated branches bore 5–7 figs larger than 0.4 cm. on May 23. Randomization of treatments was not attempted, the branches being used as found. To prevent pollination by the fig-wasp, all selected branches were bagged on May 23 (whether they were treated on that date or later) with white muslin bags commonly used for this purpose, and remained covered until June 24. Three branches were used as replicates for each treatment, except for the oil emulsion sprays, where only two were used.

At the time of the first treatment, and at frequent intervals thereafter, measurements were made of the transverse diameter (in mm.) of each treated fig on each branch, numbering the figs from base to tip of the branch. In addition, observations were made on the color and shape of the treated figs, and on the condition of the foliage. As controls, 10 untreated and unbagged branches, scattered through the various treatments, were measured and observed. In all, about 175 branches were used on 40 trees, and the development of over 1000 figs was followed for a period of 90 days.

Results and discussion

The injection of water solutions or suspensions of growth-regulators into the fig cavities through the ostioles failed, with three exceptions, to induce fruit set, and all such figs shriveled and dropped from the trees at the same time as uncaprified control figs. The exceptions to these results were IBA at both concentrations and NOA + IBA at the lower concentration injected into syconia toward the end of the caprification period (June 12). From this treatment several of the figs at nodes 1 to 3 reached normal size and were mature, but seedless, just 12 days later on June 24. This was a phenomenal response as evidenced by the fact that the caprified con-

trol fruits did not mature until the first week in August. On the other hand, parthenocarpic fruits, induced as a result of spray treatments, were slower to mature than the caprified control fruits, as will be discussed later. The significance of this abnormally rapid fruit development is not understood as yet.

No apparent foliage or twig injury was present on any of the branches bearing syconia that were injected. Spraying the branches, figs, and foliage with the two different concentrations of 2,4-D and NOA likewise caused no foliage or twig injury. However, the use of IBA, either alone or in combination with NOA, caused moderate leaf curl and moderate to severe chlorosis, a condition that persisted throughout the season until leaf drop.

Of the various spray treatments, 2,4-D and NOA in water solutions at both concentrations failed to set fruit. Likewise, NOA alone and in combination with IBA in an oil emulsion failed to stimulate fruit development. In other words, parthenocarpic fruit development resulted only from aqueous spray applications of both concentrations of IBA alone and in combination with NOA and the oil emulsion spray containing IBA at a concentration of 2670 p.p.m.

It should be pointed out that 2,4-D, when injected or sprayed, gave some indication of being able to set figs parthenocarpically. Several days after treatment, syconia treated with this material were definitely further along in development than the controls, as measured by shape and color. Although all of the syconia treated with 2,4-D eventually dropped when about half grown, for a time they exhibited all the appearances of being set in that their color changed from a dark green to a light yellowish green and all ridges had disappeared as a result of swelling. Higher concentrations or repeated applications of the material to the same figs might possibly result in parthenocarpic development.

Treatments that were effective in inducing parthenocarpic fruit development are set forth in table I, together with the average percentage fruit set with each treatment for the different times of application. The average percentage fruit set with the three applications of each chemical was from 15 to 20 per cent. higher than fruit set in the normally caprified controls, with exception of the aqueous IBA (2670 p.p.m.) treatment which was about equal to the controls. These data indicate that no benefit was derived from combining NOA with IBA. The apparent inadequacy of NOA at the concentrations used is further corroborated by the fact that when used alone and applied as an injection or spray it failed to induce fruit set. It is evident, likewise, that 1500 p.p.m. of IBA is just as effective as applications of the same material at a concentration of 2670 p.p.m. Perhaps concentrations considerably lower than 1500 p.p.m. would prove to be highly effective in inducing parthenocarpic fruit set in the fig.

Data in table I suggest that there is a relatively short period of time during which there is a maximum number of syconia on the tree that are

in a receptive condition for pollination or for being set parthenocarpically. Although the time between the first and second and the second and third applications of a particular preparation was only 14 and 8 days, respectively, in general, the treatments applied about the middle of the caprification period resulted in a much higher percentage of fruit set than treat-

TABLE I

RELATION OF TIME OF GROWTH-REGULATOR APPLICATION DURING THE CAPRIFICATION PERIOD TO THE PERCENTAGE FRUIT SET FOR EACH TREATMENT

TREATMENT AND TIME OF APPLICATION*		FRUIT SET
		%
Indolebutyric acid (2670 p.p.m.) in water	Beginning	41.2
	Middle	60.0
	End	53.8
	Average	51.1
Indolebutyric acid (1500 p.p.m) in water	Beginning	66.7
	Middle	70.6
	End	75.0
	Average	70.6
Naphthoxyacetic acid (100 p.p.m.) and indolebutyric acid (2670 p.p.m.) in water	Beginning	66.7
	Middle	82.4
	End	52.9
	Average	67.3
Naphthoxyacetic acid (50 p.p.m.) and indolebutyric acid (1500 p.p.m.) in water	Beginning	50.0
	Middle	88.2
	End	60.0
	Average	66.7
Indolebutyric acid (2670 p.p.m.) in oil emulsion	Beginning	72.7
	Middle	90.9
	End	50.0
	Average	71.9
Check (caprified)	Average	51.7

* Time in relation to caprification period: Beginning—5/23/47, Middle—6/5/47, End—6/12/47.

ments applied either before or after. Since the fig bears its fruits laterally in the axils of leaves on current season's wood, each successive fig on the shoot is somewhat later in development to the one it succeeds. The percentage fruit set for figs sprayed with an oil emulsion containing 2670 p.p.m. of IBA indicates that at a particular time as many as 90 per cent. of the figs are in a receptive condition or, at least, in a state whereby they can be set parthenocarpically.

Data in table II, on the relation of the time of application to the percentage fruit set at the various nodal positions, show that, in general, all

treatments applied at the beginning of the caprification period set fruits at nodes one through four. The same treatments applied about the middle of the caprification period showed a tendency to set fruits at all of the nodal positions, while applications near the end of this period set the greatest percentage of fruits toward the end of the new shoots. Appar-

TABLE II

RELATION OF TIME OF GROWTH-REGULATOR APPLICATION DURING THE CAPRIFICATION PERIOD TO THE PERCENTAGE FRUIT SET AT THE DIFFERENT NODE POSITIONS

TREATMENT AND TIME OF APPLICATION*		NODE POSITION†					
		1	2	3	4	5	6
Indolebutyric acid (2670 p.p.m.) in water	Beginning	100	100	33	0	0	0
	Middle	100	67	67	67	0	.....
	End	0	0	100	100	100	.....
Indolebutyric acid (1500 p.p.m) in water	Beginning	100	100	100	100	0	0
	Middle	67	33	100	67	67	100
	End	0	67	100	100	100	100
Naphthoxyacetic acid (100 p.p.m.) and indolebutyric acid (2670 p.p.m.) in water	Beginning	100	100	100	100	0	0
	Middle	100	100	100	100	67	0
	End	0	0	33	100	100	100
Naphthoxyacetic acid (50 p.p.m.) and indolebutyric acid (1500 p.p.m.) in water	Beginning	67	100	33	33	33	0
	Middle	100	100	100	100	67	50
	End	0	33	67	100	100	.....
Indolebutyric acid (2670 p.p.m.) in oil emulsion	Beginning	100	100	100	100	0	0
	Middle	100	100	100	50	100	50
	End	0	50	50	50	100	100
Check (caprified)		0	60	50	80	60	50

* Time in relation to caprification period: Beginning—5/23/47, Middle—6/5/47, End—6/12/47.

† Nodes numbered 1 to 6 beginning at base of current season's growth.

ently syconia at nodes five and six had not yet reached a receptive condition when the first treatments were made, whereas the picture was reversed as regards the last treatment when the syconia at nodes one and two had apparently passed this point. It is interesting to note that at the first node position on the control branches not a single fig was set. Evidently figs at this position had passed their receptive state for pollination before the caprifigs were suspended in the trees. Likewise, it is significant to note that in no case was there complete set of fruit at any of the various nodal positions on the check branches. The significant fact of the data in table II is that by the correct timing of the growth-regulator application it appears possible to obtain 100 per cent. set of fruit. Perhaps this might be accomplished by two successive treatments but the data indicate that one application at the critical time might result in a complete set of fruit.

Periodic cross-diameter measurements of caprified control fruits and chemically-induced parthenocarpic fruits are represented in figure 1 by

typical curves of growth for these two types of figs. Attention is called to the fact that the fig fruit, like stone or drupaceous fruits, goes through a double cycle of growth which may be represented by a double sigmoid curve. Development of the peach fruit was segregated by CONNORS (7) into three definite stages of growth: stage I, rapid rate of growth following fertilization; stage II, a period of rest during which time the seed develops; and stage III, accelerated growth rate of the flesh to maturity. In

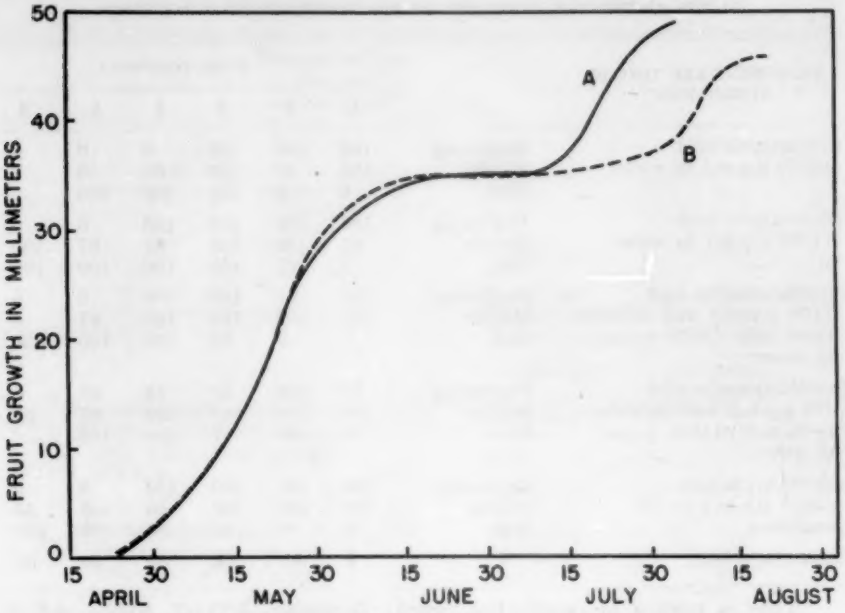

FIG. 1. Typical curves of growth in diameter or caprified (A) and growth-regulator-induced (B) parthenocarpic Calimyrna fig fruits.

figure 1 it is shown that the rates of growth during most of stage I were identical for those fruits that were destined to develop parthenocarpically and those that were to produce seeds as a result of caprification. Shortly after the sprays were applied on May 24, the treated figs grew at a somewhat more rapid rate but later ceased enlargement in stage II at the same average diameter as figs that were caprified. A difference of about 20 days in the length of time the two types of fruit remained in the resting stage, or stage II, was responsible for the fact that parthenocarpic fruits did not mature until somewhat after maturation of fruits that were caprified. Similar observations on maturity have been made on the Kadota variety which is completely parthenocarpic in its development. When a caprifig tree is in close proximity to a Kadota orchard, wasps drift in on the wind and caprify occasional syconia that invariably ripen about two weeks in advance of fruits that develop parthenocarpically.

In the Calimyrna variety, the chemically-induced parthenocarpic fruits

were not markedly different, in external characteristics, from fruits that were caprified. Mature caprified fruits had an average diameter of 46 millimeters as compared with 48, 44, 50, 47, and 44 millimeters for parthenocarpic fruits induced by aqueous applications of IBA at 2670 and 1500 p.p.m., NOA 100 p.p.m. + IBA 2670 p.p.m., NOA 50 p.p.m. + IBA 1500 p.p.m., and by an oil emulsion containing IBA at 2670 p.p.m., respectively. The two types of fruit exhibited about the same degree of color intensity, both being typically golden yellow. As shown in figure 2, the neck of the caprified fig is typically compressed and flattened laterally and joins the body abruptly, while the necks of the parthenocarpic figs taper more gradually from stalk to body.

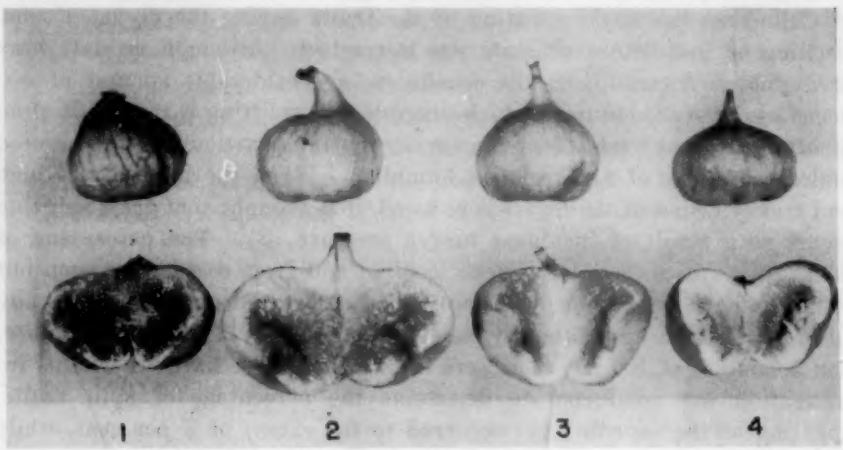

FIG. 2. Typical caprified and growth-regulator-induced parthenocarpic Calimyrna fig fruits. 1, caprified; 2, indolebutyric acid (1500 p.p.m.) in water, applied 5/24/47; 3, naphthoxyacetic acid (50 p.p.m.) and indolebutyric acid (1500 p.p.m.) in water, applied 6/12/47; and 4, indolebutyric acid (2670 p.p.m.) in water, applied 6/5/47. No significance should be attached to the difference in fruit sizes.

The most striking internal difference between caprified and parthenocarpic Calimyrna figs is the complete absence of seeds (achenes) in the latter (fig. 2). Figs, such as the Mission and Brown Turkey, which are completely parthenocarpic, are characterized by having numerous hollow and infertile achenes with the ovary wall fully sclerified. In the chemically-induced parthenocarpic Calimyrna figs, however, there was no sclerification of the ovary wall.

According to CONDIT (6), the pulp of the fig is composed of the inner part of the meat, the perianth and floral peduncles, the parenchymatous outer cell walls of the ovaries, and the seed. The parenchymatous tissue surrounding the floral organs becomes greatly enlarged and serves as storage tissue. As the flowers mature, they may completely fill the cavity and form a solid pulp as is characteristic of caprified Calimyrna figs. In some

instances, floral organs in the induced parthenocarpic figs did not enlarge to the same degree as those in the caprified figs, leaving their cavities incompletely filled. Repeated applications of the growth-regulator to the same fruit might possibly correct this condition.

The pulp of the caprified figs was light strawberry in color while that of the parthenocarpic figs was light amber yellow. The flavor of the two types of figs was the same, so far as could be detected. Apparently the lack of seeds did not detract from the flavor of the parthenocarpic figs. Sugar analyses showed that the dried caprified figs contained 30.0 per cent. sugar and the dried parthenocarpic fruits 27.4 per cent., all the sugar being of the reducing form in both cases.

A problem of rather serious consequence accompanying the production of Calimyrna figs is the splitting of the fruits during the period of final swelling or just before ultimate size is reached. Although no data have been published to support the conclusion, a considerable amount of evidence has been accumulated which suggests that splitting is the result of an inherent physical weakness of the variety which is particularly pronounced under conditions of high relative humidity. When the humidity is high and transpiration of the leaves is reduced, it is thought that fruit splitting occurs as a result of increased turgor pressure (**3**). The percentage of split fruits varies from location to location and from season to season but instances have been reported where splitting had occurred in practically all of the mature figs examined in several orchards (**12**). In this connection 5 samples of 100 figs each were counted in the orchard where this investigation was conducted to determine the percentage of split fruits. Splitting of the caprified figs occurred to the extent of 5 per cent. while no splitting was found to have taken place on any of the parthenocarpic figs. These observations indicate that splitting may be the result of a growth phenomenon associated with seed development which occurs, as pointed out above, under certain environmental conditions.

A method that would eliminate the costly, uncertain, and potentially disease-spreading caprification techniques, and at the same time maintain quality and quantity of yield, would be of great material benefit to the Calimyrna fig grower. The present results indicate that such a method is within the realm of possibility.

Summary

1. This paper presents the preliminary results of investigations on the chemical induction of parthenocarpic development in the Calimyrna fig. This variety, as well as all varieties belonging to the Smyrna group, requires cross-pollination in order for the syconia to set and mature.

2. Aqueous sprays containing indolebutyric acid at concentrations of 1500 and 2670 p.p.m., and an oil emulsion spray containing 2670 p.p.m. of indolebutyric acid resulted in parthenocarpic fruit set that was equal to or better than the caprified (pollinated) controls. Neither naphthoxy-

acetic nor 2,4-dichlorophenoxyacetic acids at the concentrations used were effective in promoting parthenocarpy. No apparent foliage injury resulted from applications of 2,4-dichlorophenoxyacetic or naphthoxyacetic acids but indolebutyric acid caused moderate curling of the foliage accompanied by chlorosis which persisted throughout the season.

3. A higher percentage of figs were set when treatments were made about the middle of the caprification period than when they were made either at the beginning or toward the end of this period. By applying an aqueous spray of indolebutyric acid at the proper time, the data presented indicate that it may be possible to set parthenocarpically nearly all the syconia on the tree.

4. The injection of these growth regulators through the ostioles and into the cavities of figs, produced no consistent parthenocarpic development. However, some figs treated in this manner reached full maturity in 12 days, as compared to approximately 80 days for caprified and 95 days for parthenocarpic figs induced through spraying.

5. Parthenocarpic figs were not markedly different from caprified fruits as regards color, size, shape, flavor, and sugar content, but they were completely seedless with no sclerification of the ovary walls.

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UNIVERSITY OF CALIFORNIA
DIVISION OF POMOLOGY
DAVIS, CALIFORNIA
AND
SHELL OIL COMPANY, INCORPORATED
AGRICULTURAL LABORATORY
MODESTO, CALIFORNIA

LITERATURE CITED

1. BURKHOLDER, C. L., and MCCOWN, MONROE. Effect of scoring and of a-naphthyl acetic acid and amide spray upon fruit set and of the spray upon pre-harvest fruit drop. Proc. Amer. Soc. Hort. Sci. **38**: 117–120. 1941.
2. CALDIS, PANOS D. Etiology and transmission of endosepsis (internal rot) of the fruit of the fig. Hilgardia **2**: 287–328. 1927.
3. COIT, J. E. Fig splitting and souring can be reduced. Pacific Rural Press **101**: 300. 1921.
4. CONDIT, IRA J. Parthenocarpy in the fig. Proc. Amer. Soc. Hort. Sci. **36**: 401–404. 1938.
5. ————————. Caprifigs and caprification. California Agr. Exp. Sta. Bul. **319**. 1920.
6. ————————. The structure and development of flowers in *Ficus carica* L. Hilgardia **6**: 443–481. 1932.

7. CONNORS, C. H. Growth of fruits of peach. New Jersey Agr. Exp. Sta. Ann. Rept. **40**: 82–88. 1919.
8. GREENE, LAURENZ. Growth regulators and fruit set with Starking apples. Proc. Amer. Soc. Hort. Sci. **42**: 149–150. 1943.
9. GUSTAFSON, F. G. Inducement of fruit development by growth-promoting chemicals. Proc. Nat. Acad. Sci. **22**: 628–636. 1936.
10. KOLESNIKOW, V. A. Parthenocarpy and self-pollination in fruit. (trans. title). Bulgarian Salgier Pomol. Exp. Sta. **2**: 3–42. 1927.
11. POMEROY, C. S., and ALDRICH, W. W. Set of citrus fruits in relation to applications of certain growth substances. Proc. Amer. Soc. Hort. Sci. **42**: 146–148. 1943.
12. SMITH, R. E., and HANSEN, H. N. Fruit spoilage diseases of figs. California Agr. Exp. Sta. Bul. **506**. 1931.
13. ————————. The improvement of quality in figs. California Agr. Exp. Sta. Cir. **311**. 1927.
14. STOUT, A. B. Seedlessness in grapes. New York (Geneva) Agr. Exp. Sta. Tech. Bul. **238**. 1936.
15. VARRELMAN, F. A. Anent parthenocarpic apples. Science **87**: 414. 1938.
16. WITHROW, R. B., and HOWLETT, F. S. New carriers for plant growth regulators. Plant Physiol. **21**: 131–139. 1946.

RELATION BETWEEN ROOT RESPIRATION AND ABSORPTION

C. C. WILSON AND P. J. KRAMER

(WITH TWO FIGURES)

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The rate of absorption of water by roots of transpiring plants is in general closely dependent upon the rate of transpiration, and a change in the rate of transpiration is soon followed by a corresponding change in the rate of absorption (**3**). Since the rate of absorption lags behind and apparently is dependent on the rate of transpiration it is usually considered that under conditions such that transpiration exceeds absorption the roots play a passive role in the absorption of water. It has been reported by certain workers, however, that a close relation exists between root respiration and water absorption. NEWTON (**6**) observed that the rate of root respiration was increased when the concentration of the culture solution in which the roots were immersed was increased. He suggested that this was caused by the greater expenditure of energy required to absorb a given volume of water from a more concentrated solution. HENDERSON (**1**) reported that there was a direct relation between respiration and water absorption in the roots of transpiring corn seedlings and concluded that the absorption of water was brought about by the expenditure of energy released in respiration. LOWENECK (**5**), on the other hand, found no relation between root respiration and rate of transpiration of *Zea maize* or *Sonchus oleraceus*. In view of these differences in opinion it seemed desirable to make a further study of the problem, using large plants.

Tomato seeds of the Marglobe variety were germinated in moistened quartz sand and the seedlings were transferred to solution cultures. The plants were grown in an unshaded greenhouse until approximately two months old. The most vigorous plants were then selected and sealed at the level of the crown into 250 ml. potometers. Four potometers were placed in a constant-temperature water bath and were connected to a common source of aerated tap water. The oxygen content of the water in the potometers was determined by the Winkler method, determinations being made before and after each hour exposure of the plants to differing environmental conditions. Since all four potometers were connected to the same source of aerated water, the four root systems were under similar conditions, as indicated by the fact that the determinations of oxygen content at the beginning of each experimental period were always in close agreement. Nearly 100 determinations of the absorption of oxygen and water were made under conditions producing widely differing rates of transpiration and hence of water absorption. The results are presented in table I.

The relation between rate of respiration as measured by the absorption of oxygen, and rate of absorption of water is shown graphically in figure 1, where data from four plants are presented separately. In no graph is there any obvious relation between rate of respiration and rate of water absorption. A regression equation was calculated from the data for

TABLE I

RATES OF ABSORPTION OF WATER AND OXYGEN BY TOMATO ROOTS IN C.C.'S./HR.

PLANT I		PLANT II		PLANT III		PLANT IV	
c.c. H_2O ABSORBED	c.c. O_2 ABSORBED	c.c. H_2O ABSORBED	c.c. O_2 ABSORBED	c.c. H_2O ABSORBED	c.c. O_2 ABSORBED	c.c. H_2O ABSORBED	c.c. O_2 ABSORBED
3.40	2.12	3.20	1.66	2.20	0.47	3.60	1.01
2.50	1.78	2.40	1.74	1.20	0.76	2.20	1.06
1.30	1.31	0.75	1.27	0.50	0.64	0.70	0.85
1.10	1.21	0.50	1.69	0.40	0.70	0.50	1.14
0.50	0.72	0.30	0.79	1.02	2.42	0.58	0.84
0.27	1.17	0.28	1.26	0.80	2.57	0.17	1.11
1.37	1.08	0.26	0.70	0.70	2.96	0.12	1.29
2.50	1.15	1.53	0.46	2.46	1.60	0.63	0.38
3.01	0.87	1.78	0.71	2.97	2.37	0.96	0.17
3.90	1.15	1.96	0.29	3.01	1.82	1.25	0.58
4.20	0.97	1.97	0.29	2.87	1.63	0.90	0.29
3.06	1.14	2.09	0.43	3.06	1.59	1.45	0.52
4.90	0.74	1.73	0.45	2.48	1.16	1.14	0.34
2.84	0.77	0.26	0.52	0.34	1.18	0.30	0.68
1.10	1.35	0.31	0.40	0.51	1.59	0.41	0.91
3.90	2.35	2.90	2.14	2.30	0.92	3.40	1.33
4.20	1.85	3.40	1.70	1.20	0.54	4.00	1.30
0.85	1.30	0.19	0.74			0.12	0.74
2.85	0.91	0.75	0.89			2.86	0.55
2.15	1.25	0.85	1.01			0.73	0.50
2.20	1.19	1.04	0.87			0.95	0.61
2.40	1.45	1.05	1.01				
3.45	1.32	1.00	1.09				
3.60	1.31	1.02	1.07				
3.58	1.20	1.12	0.64				

each plant, and the regression coefficients were tested by an analysis of variance and found not to be significant. This statistical analysis of the data failed to establish any definite relation between rate of respiration and rate of water absorption.

Regression equations were also calculated for the data obtained by Henderson from her plants, numbers 8, 9, 10, and 11, for which the rate of root respiration, as measured by absorption of oxygen, was related to the absorption of water. These regression coefficients were also found not to be significant, indicating that no definite relation existed between the rate of respiration of the corn roots studied by Henderson and their absorption of water. Although this conclusion seems to be at variance with the graphs in Henderson's paper illustrating the parallelism between respiration and

absorption, a careful study of her data indicates that for any given rate of respiration almost any absorption rate can be found (fig. 2).

Apparently Henderson's conclusion that water intake and root respiration of corn seedlings were correlated was not actually supported by her

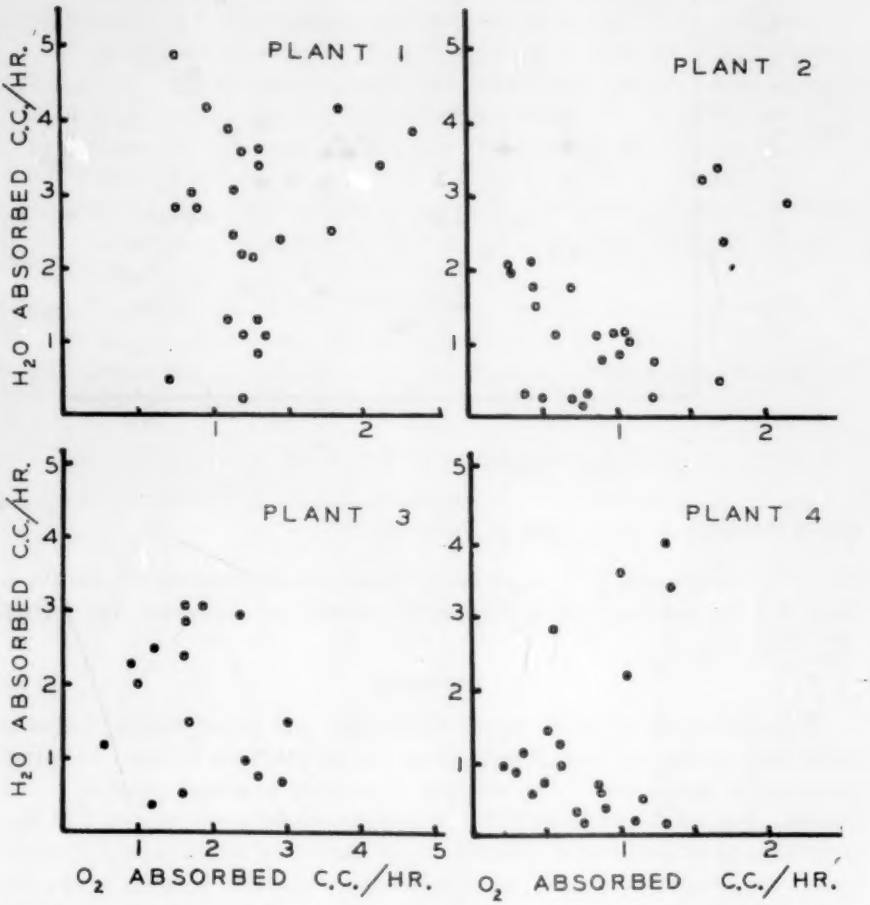

Fig. 1. Relation between rate of respiration as measured by absorption of O_2, and rate of absorption of water.

own experimental data. Neither did such a correlation exist in the large tomato plants studied by the writers. It seems quite unlikely that any such correlation could exist in freely transpiring plants, because in such plants absorption is determined by the rate of water loss rather than by the expenditure of energy by the root cells (**4**). This conclusion does not apply, of course, to the intake of water by isolated pieces of plant tissue or by root systems of plants with very low transpiration rates. Much evidence has been accumulated indicating that in such material water intake

is at least partly related to the release of energy in respiration (7). For instance, intake of water by root systems and pieces of plant tissue is re-

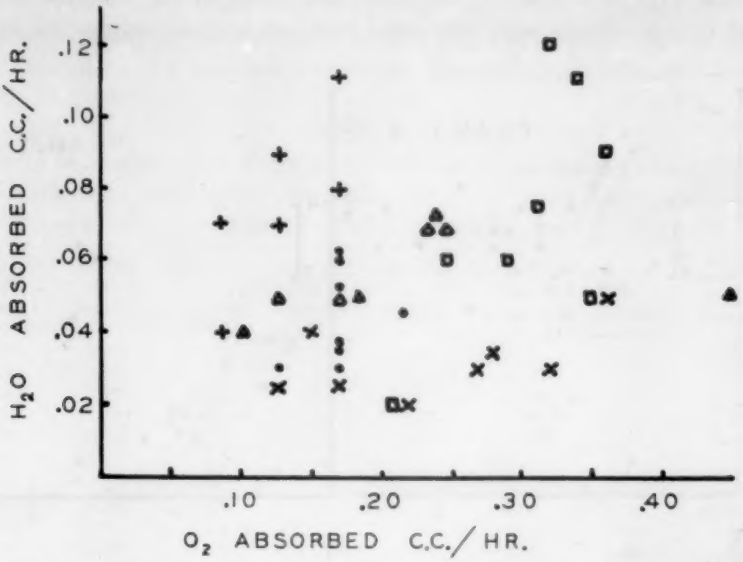

Fig. 2. Relation between rate of respiration as measured by absorption of O_2. and rate of absorption of water. Data of HENDERSON (1).

duced by substances such as cyanide which inhibit respiration, and it is increased by indoleacetic acid, possibly because of increased respiration (2) (8).

Summary

Respiration, in terms of oxygen absorption, and water intake of tomato roots were measured concurrently while the shoots were exposed to varied atmospheric conditions which resulted in a wide range of rates of water intake. No definite relation could be established between rate of root respiration and rate of water absorption. A statistical analysis of data published by Henderson showed no significant correlation between water absorption and root respiration. It seems probable that the energy released by respiration plays no significant role in the intake of water through the roots of freely transpiring plants.

UNIVERSITY OF GEORGIA
ATHENS, GEORGIA
AND
DUKE UNIVERSITY
DURHAM, NORTH CAROLINA

LITERATURE CITED

1. HENDERSON, L. Relation between root respiration and absorption. Plant Physiol. **9**: 283–300. 1934.
2. KELLY, S. The relationship between respiration and water uptake in the oat coleoptile. Amer. Jour. Bot. **34**: 521–526. 1947.
3. KRAMER, P. J. The relation between rate of transpiration and rate of absorption of water in plants. Amer. Jour. Bot. **24**: 10–15. 1937.
4. LEVITT, J. The thermodynamics of active (non-osmotic) water absorption. Plant Physiol. **22**: 514–526. 1948.
5. LOWENECK, N. Untersuchungen über Wurzelatmung. Planta **10**: 185–228. 1930.
6. NEWTON, J. R. The relation of the salt concentrations of the culture solution to transpiration and root respiration. Sci. Agr. **5**: 316–320. 1925.
7. VAN OVERBEEK, J. Water uptake by excised root systems of the tomato due to non-osmotic forces. Amer. Jour. Bot. **29**: 677–683. 1942.
8. VAN OVERBEEK, J. Auxin, water uptake and osmotic pressure in potato tissue. Amer. Jour. Bot. **31**: 521–526. 1947.

EFFECTS OF 2,4-D ON INBRED LINES AND A SINGLE CROSS OF MAIZE[1]

ELMER C. ROSSMAN AND DAVID W. STANIFORTH

(WITH FIVE FIGURES)

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Introduction

The use of 2,4-dichlorophenoxyacetic acid and other growth regulating chemicals as selective herbicides for weed control in crop fields has increased rapidly in the last three years. 2,4-D, particularly, has been advocated for selective control of broad-leaf weeds in grasses, including cereals. The herbicide may, however, injure the crop under certain developmental or climatic conditions. Varietal differences also may account for some of the variable effects reported for corn.

In general, the cooperative reports from several experiment stations (**6**) indicate that applications of 2,4-D to foliage of corn in the early stages of growth may reduce yields. Of the cereal crops, corn seemed the most sensitive. BAKKE and SYLWESTER (**2**) have stated that corn may be damaged if sprayed before the ears have reached the milk stage. Pre-treatment of the soil with 2,4-D resulted in a stimulation of corn growth in experiments conducted by TAYLOR (**11**). Evidence that inbred lines and hybrids may differ in percentage of affected seedlings when pre-emergence treatments of 2,4-D were made to the soil has been presented by ANDERSON and WOLF (**1**).

PRIDHAM (**10**) reported that the seedlings of red kidney beans from parents sprayed with 2,4-D during the ripening of pods showed characteristic 2,4-D injury. GRIGSBY (**5**) found that pollen production and floral development of ragweed were reduced or inhibited when sprayed with several of the new herbicidal compounds. According to the cooperative reports of the NORTH CENTRAL WEED CONTROL CONFERENCE (**6**), test weight and germination of grain from treated oats, barley, or wheat differed little from untreated checks except in cases where plants showed severe injury as a result of spraying. MARTH *et al.* (9) reported that seed stalks and seed heads developed on 2,4-D treated timothy seemed unaffected, and that the germination of seed developed on plots treated in various ways was unaffected. Application of 2,4-D to the soil had no adverse effects on the viability of Kentucky bluegrass seed, but a measurable effect was found in reducing seed dormancy (**8**). In the classification of crop plants as to

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their reaction to 2,4-D when treated in an actively growing stage (**12**), corn is listed in the "intermediate" group with barley, oats, wheat, and rye, while the "resistant" crop plants include most hay and pasture grasses such as bromegrass, timothy, ryegrass, orchard grass, Kentucky bluegrass, Canada bluegrass, and Bermuda grass.

The work reported here was designed to test possible varietal and developmental differences in the responses of corn to 2,4-dichlorophenoxyacetic acid, and to study the possibility of deleterious effects in seed produced on treated parental plants and in plants of the succeeding generation.

Materials and methods

Inbred lines

Four inbred lines of corn, M14, WF9, Os420, and W22 were grown in 25 plant nursery rows. The aerial portions of the plants were sprayed with aqueous solutions of 400 p.p.m. or 1600 p.p.m. of the sodium salt of 2,4-D at three stages of plant development: (1) six-eight leaves, (2) tassel and ear shoot emergence, and (3) ten days after pollination. The plots were sprayed on July 3, August 8, and August 30, 1947. A knapsack sprayer was used which delivered approximately 75 gallons per acre for the first date and 150 gallons per acre for the last two dates. Twenty-eight rows of each inbred line were grown; a different block of eight rows was sprayed on each of the three dates and four rows were left as unsprayed checks. Applications were made on warm bright days and no rain fell for at least four days after any of the spraying dates. The corn growing season at Ames, Iowa, 1947 was extremely wet during May and June with hot, dry weather in July and August. Total rainfall for July and August was approximately 1.5 inches.

Notes on the percentage of plants showing visible effects of the treatments were taken after the first spraying. Plants sprayed on July 3 were counted as affected when there was definite evidence of stalk curvature on July 11 and of leaf rolling on July 21. On August 21, data were taken on the percentage of plants that had produced either no tassels or a barren rachis with no staminate inflorescences.

Hand pollinations were made to give seed of all possible single crosses, including reciprocals, among the four inbred lines within each rate and date of treatment. Seed of the 12 single crosses, with seven treatments (including check) applied to each, was harvested and dried in a seed-corn drier. Seventy-seven of the 84 possible treatment combinations were planted in sand in a greenhouse bench in a split-plot design with five replications of 25 seeds per plot. Sixteen days after planting counts were made for total germination and weak seedlings, and green weights of the tops cut at the ground level were obtained.

Single-cross hybrid

Plants of a single-cross hybrid (WF9 × 38–11), grown in 20 hill plots,

were treated with the sodium salt of 2,4-D in aqueous solution using a knapsack sprayer, according to the following schedule. (1) Concentrations of 400 p.p.m. or 1600 p.p.m. were applied at the rate of 65 gallons per acre when the plants were 18–20 inches tall (early date). (2) The same concentrations were applied at the rate of 165 gallons per acre to previously untreated plots when 70–80 per cent. of the plants were in the silk stage and pollen was abundant (late date). (3) Applications of 400 p.p.m. were made to another set of plots at both the early and late dates.

The effect of adding Dreft, a commercial detergent, one-half per cent. by weight to 2,4-D water solutions was tested in a series of plots. (1) Plants three to four feet tall, with no tassels emerged, were sprayed in triplicate plots with 400 p.p.m. or 1600 p.p.m. of 2,4-D with and without Dreft added, and with Dreft alone. (2) Solutions of 2,4-D containing one-half per cent. Dreft were applied to leaves of several plants with a cloth at three stages of growth: (a) 1600 p.p.m. or 3200 p.p.m. four to five days before silks emerged, (b) 800 p.p.m. or 1600 p.p.m. when silks were emerging, and (c) 1600 p.p.m. ten days after silks had emerged.

Experimental results

Effects on inbred lines

Vegetative responses.—Applications of 2,4-D to the foliage of the four inbred lines at the two later stages of plant development, tassel emergence and ten days after pollination, produced no visible effects on any of the above-ground plant parts. Therefore, the results on vegetative responses discussed below apply only to the plots treated in the six-eight leaf stage.

In table I are given data on the percentage of plants affected when

TABLE I

Percentage of plants visibly affected on July 11, July 21, and August 21 by 400 p.p.m. or 1600 p.p.m. of 2,4-D applied at the six-eight leaf stage. Mean of 4 plots, approximately 90 plants

Line Inbred	Per cent. affected plants on						Per cent. defective tassels		
	July 11 Stalk curvature			July 21 Leaf rolling			August 21		
	Rate p.p.m.			Rate p.p.m.			Rate p.p.m.		
	400	1600	Check	400	1600	Check	400	1600	Check
M14	0.0	11.1	0.0	13.7	65.9	0.0	0.0	11.0	0.0
WF9	0.0	8.1	0.0	1.0	8.0	0.0	0.0	0.0	0.0
Os420	5.6	24.1	0.0	1.1	2.4	0.0	0.0	0.0	0.0
W22	12.3	62.2	0.0	9.2	100.0	0.0	22.9	65.8	0.0

sprayed in the first stage. The data taken on July 11 represent the percentage of plants exhibiting marked stalk curvature, since this was the predominant response one week after treatment. This response is illustrated

FIG. 1. (A) W22 plants on July 11 when 1600 p.p.m. 2,4-D sprayed plot on left and unsprayed plot on right. (B) W22 plants on July 19 with 2,4-D, 1600 p.p.m. 2,4-D, and unsprayed check. (C) and (D) Roots of W22 and M14 on July 20. Left to right, 400 p.p.m. was applied on July 3, 1947.

in figure 1 A. Inbred W22 exhibited more stalk curvature than the other three lines. By July 21 most of the plants had reassumed an upright position, and extreme leaf rolling, as shown in figure 1 B and figure 2, was the characteristic visible response. M14 and W22 showed 65.9 per cent. and

FIG. 2. Four inbred lines of corn sprayed with 1600 p.p.m. 2,4-D on July 3, 1947. Pictures taken July 19. (A) W22, (B) M14, (C) Os420, (D) WF9.

FIG. 3. (A) W22 plant on August 21. Note barren rachis of tassel. (B) M14 plant with practically no tassel. Both sprayed with 1600 p.p.m. 2,4-D on July 3, 1947.

100.0 per cent., respectively, of affected plants in the heavy rate of application, while WF9 and Os420 showed no visible effects at this date or throughout the season. Survival of treated plants was 90 per cent. or more in all of the plots, and a considerable portion of the leaf rolling disappeared as the season advanced.

The data for percentage of defective tassels counted on August 21, table I, show that M14 and W22 were susceptible to tassel injury when sprayed

TABLE II

YIELD OF SHELLED SEED AS A PERCENTAGE OF CHECKS FOR FOUR INBRED LINES OF CORN SPRAYED AT THREE DIFFERENT STAGES OF PLANT DEVELOPMENT WITH 400 P.P.M. OR 1600 P.P.M. OF 2,4-D

INBRED LINE	STAGE WHEN SPRAYED						MEANS
	6–8 LEAVES		TASSEL EMERGENCE		10 DAYS AFTER POLLINATION		
	RATE P.P.M.		RATE P.P.M.		RATE P.P.M.		
	400	1600	400	1600	400	1600	
M14	78.6	49.3	68.2	18.0	95.6	91.3	66.8
WF9	36.7	23.5	91.9	23.6	86.2	65.8	54.6
Os420	52.7	22.3	94.7	40.1	92.6	86.2	64.9
W22	46.8	18.3	40.8	0.4	87.9	90.0	71.7
Mean	58.4	32.2	70.3	18.4	91.4	85.4	59.4
Mean for stages	45.3		44.4		88.4		

at the early stage, but there was no visible injury to tassel formation in WF9 and Os420. No reliable observations were made on pollen production or viability in any of the treatments. Figure 3 illustrates typical injury to M14 and W22.

Normal brace root formation in M14 and W22 was affected by spraying at the early stage, but again there was no pronounced effect on WF9 and

TABLE III

MEAN AIR DRY WEIGHT PER 100 SEEDS AS A PERCENTAGE OF THE CHECK, MEAN PERCENTAGE OF WEAK SEEDLINGS, AND MEAN GREEN WEIGHT PER SEEDLING FROM SINGLE-CROSS SEED HARVESTED FROM 2,4-D SPRAYED AND UNSPRAYED INBRED LINES OF CORN

MATERNAL PARENT	UNSPRAYED CHECK	STAGE WHEN SPRAYED						MEAN OF TREATED
		6–8 LEAVES		TASSEL EMERGENCE		10 DAYS AFTER POLLINATION		
		RATE P.P.M.		RATE P.P.M.		RATE P.P.M.		
		400	1600	400	1600	400	1600	
Air dry weight per 100 seeds as percentage of checks								
M14		98.9	95.5	104.9	108.7	97.4	97.6	100.5
WF9		79.5	75.0	78.9	79.2	76.9	78.1	78.0
Os420		82.3	82.5	91.7	95.0	94.6	85.3	88.6
W22		86.9	89.5	95.5	.....	100.2	94.5	93.5
Treatment mean		85.0	85.6	90.9	96.3	91.0	87.2	89.1
Stage mean		85.3		93.1		89.1		
Percentage of weak seedlings								
M14	2.4	17.6	15.2	19.6	29.6	10.4	12.0	16.8
WF9	2.4	38.0	60.0	22.0	40.4	13.6	17.2	29.6
Os420	2.0	59.2	39.6	39.2	55.6	46.0	45.2	47.5
W22	10.4	16.4	17.6	10.0	.....	15.6	8.4	13.6
Treatment mean	4.4	32.4	32.0	22.8	43.6	21.6	20.8	27.7
Stage mean		32.2		31.0		21.1		
Mean green weight per seedling top (grams)								
M14	0.45*	0.30	0.36	0.33	0.30	0.37	0.36	0.34
WF9	0.65	0.33	0.22	0.41	0.30	0.44	0.43	0.37
Os420	0.68	0.27	0.33	0.34	0.33	0.35	0.31	0.32
W22	0.39	0.40	0.38	0.41	.....	0.44	0.41	0.41
Treatment mean	0.54†	0.33	0.33	0.37	0.31	0.40	0.38	0.36
Stage mean		0.33		0.35		0.39		

Differences for significance between:	5% level	1% level
* any two means in body of table	0.08 gms.	0.10 gms.
† any two treatment means	0.04 gms.	0.05 gms.

Os420. The plants in figure 1, C and D, are representative of the effect on brace root formation in M14 and W22. There was a marked proliferation resulting in a gall-like formation at the first brace root node of M14, and brace roots were inhibited entirely on W22 when treated at the heavy rate.

YIELDS.—Shoot bags were removed to permit open pollination of all

plants not hand pollinated, and yields were taken as the total of hand-pollinated plus open-pollinated seed from a total of approximately eighty-four plants in four rows of each inbred line in each treatment. Yields in percentage of the unsprayed checks are given in table II. Applications of 2,4-D to the foliage at the first two stages seriously reduced seed yields.

SEED QUALITY.—The data, presented in table III, on air-dry seed weights in percentage of the checks, percentages of weak seedlings, and green weights of seedling tops are the means for the three single crosses involving a common maternal parent. Seed weights were reduced 14.7 per cent., 6.9 per cent., and 10.9 per cent. for the three dates of spraying as an average for all crosses. In contrast to the vegetative responses, seed weights of M14 and W22 combinations were affected less by 2,4-D than the seed weights of WF9 and Os420 combinations. Total germination (strong plus weak seedlings) was 90 per cent. or better and was not affected by any of the treatments; therefore, the data are not presented.

TABLE IV

ANALYSES OF VARIANCE OF TRANSFORMED DATA (ANGLES = ARC SIN $\sqrt{\text{PERCENTAGE}}$) ON PERCENTAGES OF WEAK SEEDLINGS AND OF MEAN GREEN WEIGHTS PER SEEDLING TOP

SOURCE OF VARIATION	DEGREES OF FREEDOM	VARIANCE	
		WEAK SEEDLINGS TRANSFORMED	SEEDLING WEIGHTS
Replications	4	525.1	306.9
Single crosses	11	2,356.9	554.8
Maternal parents	3	7,312.5	.....
M14 vs. W22	1	4.1	.....
M14 vs. WF9	1	2,846.1	.....
(M14 + W22) vs. (WF9 + Os420)	1	7,791.3	.....
Within maternal parents	8	498.6	.....
or			
Paternal parents	3	1,023.5	.....
Within paternal parents	8	2,857.0	.....
Error A	44	117.0	92.3
Treatments	6	5,199.8	3,405.1
400 p.p.m. vs. 1600 p.p.m.	1	1,302.6	448.4
2,4-D treated vs. check	1	65,144.2	17,249.8
Stage 1 vs. stage 2	1	41.9	221.9
Stage 1 vs. stage 3	1	3,337.3	2,036.0
Stage 2 vs. stage 3	1	2,532.8	850.3
Stage 3 vs. check	1	53,725.1	9,353.4
Treatments × single crosses	59	531.0	340.1
Error B	260	72.5	29.6

Percentages of weak seedlings were transformed to angles = arc sin $\sqrt{\text{percentage}}$. BLISS (3) and others have recommended this transformation of percentage data which are of a binomial nature. An analysis of variance, table IV, was made on the transformed data. The analysis of variance of mean green weights per seedling top also is given in table IV. A highly significant increase in percentage of weak seedlings and a highly significant reduction in mean green weight per seedling top resulted from

2,4-D treatments applied to the parental plants at all three stages of development. Increasing the concentration from 400 p.p.m. to 1600 p.p.m. produced a reduction in seedling vigor. The two earlier stages of application compared with stage three produced a highly significant increase in weak seedlings and a highly significant reduction in seedling weights. The

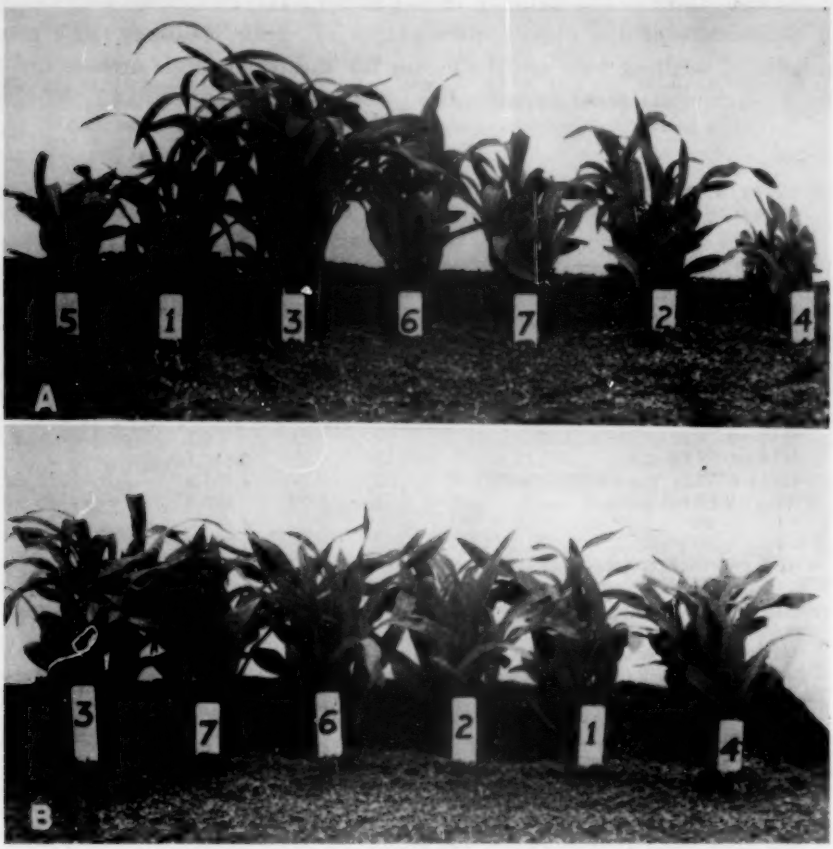

FIG. 4. Single cross seedlings from seed produced on inbred parents treated with 2,4-D at three stages of development. (A) Os420 × M14, (B) W22 × Os420. Legend: 4 = 400 p.p.m. at 6–8 leaf stage, 7 = 1600 p.p.m. at 6–8 leaf stage, 1 = 400 p.p.m. at tassel emergence, 5 = 1600 p.p.m. at tassel emergence, 2 = 400 p.p.m. 10 days after pollination, 6 = 1600 p.p.m. 10 days after pollination, 3 = unsprayed check.

interaction, treatments × single crosses, was highly significant for both weak seedlings and seedling weights, indicating that the varieties did not respond alike to the various treatments. This interaction is illustrated in figure 4. The effects of 2,4-D were much less on the single-cross seed produced on M14 and W22 than on single-cross seed produced on WF9 and Os420. This is a striking reversal of response since M14 and W22 were much more affected than WF9 and Os420 in the field when the plants were sprayed at the six-eight leaf stage.

The tests for weak seedlings and seedling vigor were repeated in flats with a mixture of two parts soil to one part sand. Essentially the same results were obtained for percentages of weak seedlings, but the differences in seedling vigor were not so extreme as when the tests were made in sand. In the plantings made in the soil-sand mixture there was an opportunity for the seedlings to become independent of the seed reserves. With favorable field conditions at planting time it might be assumed that some of the affected seedlings would recover. However, with adverse weather conditions during and following the planting season, seed quality is an important factor in obtaining good stands of corn.

Effects on a single-cross hybrid

Vegetative responses.—Single-cross plants of WF9 × 38–11, sprayed at the early date with no detergent added, showed no visible effects on any of the above-ground plant parts. There was considerable proliferation of the first ring of brace roots, similar to that in figure 1D. Spraying at the late date produced no visible effect on either the aerial parts of the plant or the brace roots.

The addition of Dreft to the spray solutions produced severe plant responses. Plants three to four feet tall exhibited severe leaf rolling and stem curvature two to three days after spraying with 400 p.p.m. or 1600 p.p.m. 2,4-D solutions with Dreft added. These responses were more pronounced with the heavy concentration. None of the plants was killed and all recovered sufficiently to produce ears which were much reduced in size.

2,4-D solutions, containing Dreft, applied with a cloth to the leaves of the single-cross hybrid plants previous to or at the time of silk emergence produced a bending in the stem at the region of the ear shoot. The stalk above the ear shoot bent at an angle of 20–25 degrees away from the vertical. Similar treatments ten days after the silks had emerged produced no stem curvature.

Yields.—Spraying the single-cross hybrid, with no detergent added to the solutions, produced no material effect on yields except for the 1600 p.p.m. concentration applied at the early stage. Applications at the late date of treatment had no effect on yield. Yield data are presented in table V. When Dreft was added to the 2,4-D solutions, yields were greatly reduced on plants sprayed when three to four feet tall, before tassel emer-

TABLE V

Yield of ear corn in pounds per 20 hill plot. Mean of six replications

Early 400 p.p.m.	Early and late 400 p.p.m.	Late 400 p.p.m.	Early 1600 p.p.m.	Late 1600 p.p.m.	Unsprayed check
19.0	17.8	19.1	16.1	19.2	19.6

gence. Sprays of 2,4-D alone and of Dreft alone did not materially reduce yields. Yield data are given in table VI.

Application of 2,4-D plus Dreft to the leaves of corn plants four to five days before the silks emerged resulted in practically no seed being formed. Similar treatments at or immediately prior to silk emergence gave variable

TABLE VI

YIELD OF CORN IN OUNCES PER EAR. MEAN OF 20–40 EARS

DREFT ALONE	UNSPRAYED CHECK	2,4-D 400 P.P.M.	2,4-D 1600 P.P.M.	2,4-D 400 P.P.M. ½% DREFT	2,4-D 1600 P.P.M. ½% DREFT
4.1	4.6	4.3	4.0	2.7	2.1

reductions in seed set. Treatments made ten days after the silks had emerged gave no reduction in seed set or in seed yield. Figure 5 illustrates the ears which were obtained from the 2,4-D plus Dreft treatments.

SEED QUALITY.—2,4-D solutions with no detergent added had no visible effect on seed quality of WF9 × 38–11. Addition of Dreft to the 2,4-D solutions resulted in a larger percentage of weak seedlings and a decrease in the green weight of seedlings compared with seed from unsprayed checks,

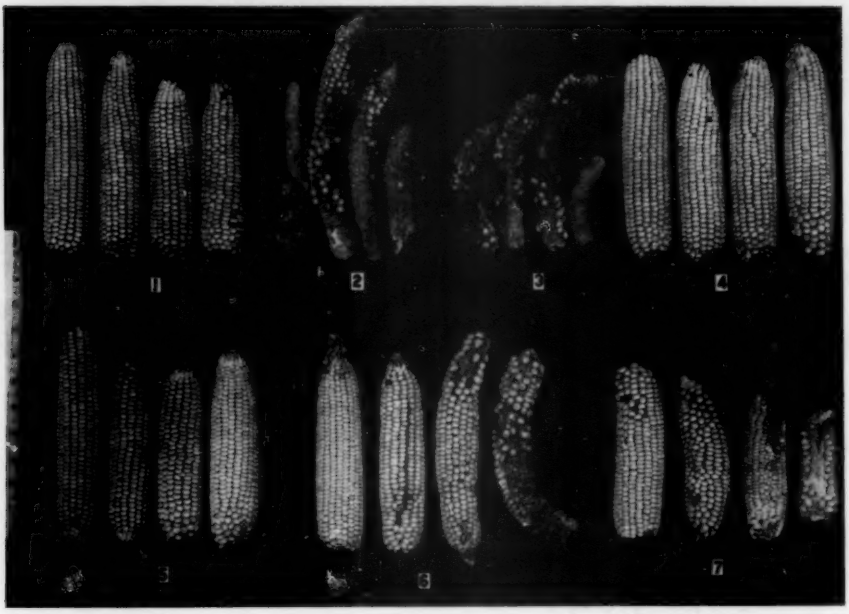

FIG. 5. Ears of corn harvested from various 2,4-D plus Dreft treatments applied to WF9 × 38–11 single cross plants. 1 = unsprayed check, 2 = 1600 p.p.m. on leaves 4 days before silks emerged, 3 = 3200 p.p.m. on leaves 4 days before silks emerged, 4 = 1600 p.p.m. on leaves 2 weeks after silks emerged, 5 = 800 p.p.m. on leaves as silks emerged, 6 = 1600 p.p.m. on leaves as silks emerged, 7 = 3200 p.p.m. on leaves as silks emerged.

except when treated ten days after pollination. In these there was no visible effect on seed quality.

Discussion

The results of this study indicate, on the basis of one year's data, that inbred lines of corn may differ in their response to 2,4-D when the foliage is sprayed at the six-eight leaf stage of development. Inbred lines may differ also in their response to 2,4-D as measured by seed quality or yields. Classification of the lines as to relative susceptibility and tolerance to 2,4-D differed with the response measured and the stage of growth when the chemical was applied. When 2,4-D was applied at the early stage M14 and W22 exhibited a greater response than WF9 and Os420, as evidenced by greater susceptibility to leaf rolling, malformation of brace roots, and defective tassel formation. ANDERSON and WOLF (1) found that seedlings of WF9 were affected less than other inbred lines of corn when pre-emergence treatments of 2,4-D as a dust were made to the soil and response measurements taken two weeks after treatment.

The difference in foliage response of the two groups of lines when treated at the early stage was not due to a lack of entrance of the 2,4-D chemical into the plant, since WF9 and Os420 showed an effect later in reduced seed yields and reduced seed quality. LUCAS and HAMNER (7) have shown that the physiological action of the sodium salt of 2,4-D may be modified by its combination with plant extracts. They found that the herbicidal activity was increased by acidification of the 2,4-D preparations, and that the effect of the 2,4-D salt was diminished in alkaline solutions. While no pH determinations of plant extracts were made on the four inbred lines in the present study, it was apparent that the physiological action of 2,4-D within the plants was different in the two groups of lines. Effects of 2,4-D on seed quality as measured by seed weights, percentages of weak seedlings, and green weights of seedling tops were the reverse of the effects on the foliage. The two lines, M14 and W22, which showed the greatest response in the foliage to 2,4-D were least affected in the seed produced. WF9 and Os420 exhibited little visible response in the foliage but were seriously affected in the seed produced. The exact nature of the difference in the physiological action of 2,4-D in the two groups of lines is not apparent from the results of this work, but differences in the destruction and translocation of the compound within the plant are indicated as possible factors.

Applications of 2,4-D at the two more mature stages of plant development, tassel emergence and ten days after pollination, produced no visible response on the vegetative parts of any of the inbred lines of corn, suggesting that the meristematic regions of the plants are more susceptible than the more mature plant tissues.

Seed yields were seriously affected by applications made at the two earlier stages, with a slight reduction when 2,4-D was applied ten days after pollination. No consistent differences were evident among the four

inbred lines of corn in the effect of 2,4-D on seed yields. Adverse growing conditions together with difficulties usually encountered in yield comparisons of inbred lines of corn may have over-shadowed any possible treatment differences among lines.

The reductions in seed weight and seedling vigor, and higher percentage of weak seedlings in seed produced on sprayed parents indicate an effect of 2,4-D on both endosperm and embryo development. The magnitude of this effect differed among the inbred lines. An examination of the data from the 12 single crosses suggests that the effect of 2,4-D on seed quality was due primarily to its effect on the maternal parent. BRITTEN (**4**) found that maize ovaries sprayed with naphthaleneacetic acid before pollination were stimulated to parthenocarpic development. Treated ovaries showed marked evidence of retardation at later stages of development, and abnormal differentiation of embryonic tissues occurred. Mature treated kernels were smaller than unsprayed kernels. He believed that carbohydrate metabolism of the developing ovaries was influenced by the treatments.

The single-cross hybrid, WF9 × 38–11, appeared to be relatively unaffected by a water spray, but the addition of a detergent to the 2,4-D solutions produced vegetative responses, yield reductions, and responses in seedlings grown from seed produced on the treated plants. The addition of Dreft reduced the surface tension of the spray solution, gave a better spread of the solution on the leaf surface, and probably facilitated the entrance of 2,4-D into the plant. Bending of the stalks of plants treated with Dreft solutions of 2,4-D was noted at all dates of treatment except when the treatment was made ten days after pollination. Seed set was not impaired by the 2,4-D plus Dreft application at the pre-tassel stage, but yields were reduced. Applications of 2,4-D plus Dreft just prior to silk emergence reduced seed set severely, whereas the same treatment ten days after pollination did not reduce seed set or yield. A possible critical time factor in the application of 2,4-D at the time of seed fertilization is indicated.

At the present time, it seems advisable to make the recommendation that 2,4-D not be applied to the foliage of foundation hybrid seed corn in the field until more information has been obtained. An experiment comparing yields of the F_1 single-cross hybrids from seed produced on the sprayed and unsprayed inbred parents in this study is planned for 1948.

Summary

1. Four inbred lines of corn, M14, WF9, Os420, and W22 were sprayed with an aqueous solution of 400 p.p.m. or 1600 p.p.m. of the sodium salt of 2,4-D at three stages of development: (1) six-eight leaves, (2) tassel and ear shoot emergence, and (3) ten days after pollination.

2. Inbred lines M14 and W22 were more susceptible than WF9 and

Os420 to rolling of leaves, inhibition of brace root development, and defective tassel formation when 2,4-D was applied to the foliage at the six-eight leaf stage. Applications made at tasseling time and ten days after pollination produced no visible effect on above-ground parts of any of the inbreds.

3. Serious reductions in seed yield occurred in all four lines when sprayed at the first two stages. Total germination of seed harvested from sprayed plants was not reduced in any of the lines, but the percentage of weak seedlings was significantly greater than in seed harvested from unsprayed plants. Seedling vigor of seed harvested from sprayed plants was significantly less than the vigor of seed harvested from unsprayed plants. Air dry weights per one hundred kernels were reduced as a result of 2,4-D applications to the parent plants.

4. The effects of 2,4-D on the percentage of weak seedlings and seedling vigor of seed harvested from sprayed plants differed with the maternal parents. The two lines, M14 and W22, which showed the greatest response to 2,4-D in the foliage were least affected in the seed. WF9 and Os420 exhibited little visible response in the field but showed the greatest effect in the seed.

5. The single-cross hybrid, WF9 × 38–11, was relatively unaffected by aqueous solutions of 2,4-D, but the addition of a detergent, Dreft, to the solutions produced vegetative response, yield reductions, and a response in the seed produced on the treated plants.

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FARM CROPS SUBSECTION, AGRONOMY SECTION, AND BOTANY AND PLANT PATHOLOGY SECTION
IOWA AGRICULTURAL EXPERIMENT STATION
AMES, IOWA

LITERATURE CITED

1. ANDERSON, J. C., and WOLF, D. E. Pre-emergence control of weeds in corn with 2,4-D. Jour. Am. Soc. Agron. **39**: 341–342. 1947.
2. BAKKE, A. L., and SYLWESTER, E. P. 2,4-D for which weeds? Iowa Farm Science **1**: 3–5. 1947.
3. BLISS, C. I. The analysis of field experimental data expressed in percentages. Plant Protection (Zashchita Rastenii) **12**: 67–77. 1937.
4. BRITTEN, E. J. The effect of naphthalene acetic acid on the developing maize caryopsis. Am. Jour. Bot. **34**: 211–217. 1947.
5. GRIGSBY, B. H. The inhibition of pollen production in ragweed by the use of chemical sprays. Science **102**: 99–100. 1945.
6. HELGESON, E. A. Uniform experiment V. Effects of various 2,4-D concentrations and formulations on crop plants. Proc. Third

Annual Meeting of the North Central States Weed Control Conference, 95–97. 1946.

7. Lucas, E. H., and Hamner, C. L. Modification of the physiological action of the sodium salt of 2,4-dichlorophenoxyacetic acid by simultaneous application of plant extracts and by pH changes. Michigan Agr. Exp. Sta. Quarterly Bulletin **29**: 255–262. 1947.
8. Marth, Paul C., Toole, Vivian K., and Toole, Eben H. Yield and viability of bluegrass seed produced on sod areas treated with 2,4-D. Jour. Am. Soc. Agron. **39**: 426–427. 1947.
9. Marth, Paul C., Toole, Vivian K., and Toole, Eben H. Influence of 2,4-D spray applications on vegetative growth and seed development in timothy. Jour. Am. Soc. Agron. **39**: 780–783. 1947.
10. Pridham, A. M. S. Effect of 2,4-D on bean progeny seedlings. Science **105**: 412. 1947.
11. Taylor, D. L. Growth of field crops in soil treated with chemical growth regulators. Bot. Gaz. **108**: 432–445. 1947.
12. Willard, C. J. Report of policy committee on herbicides. Proc. Third North Central States Weed Control Conference, 101–112. 1946.

BUFFER INDEX VALUES IN RELATION TO SOIL–pH TOLERANCES

JAMES SMALL AND TREVOR JACKSON

(WITH ONE FIGURE)

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It is now well known that some plants grow only within particular narrow ranges of soil-pH values, while other plants grow well within much wider ranges. The degrees of tolerance can be conveniently described as:— *acidiphilous,* only below pH 4.8; *mesophilous,* within the range pH 4.8 to pH 7.0; *alkaliphilous,* only above pH 7.0; *acid-tolerant,* below pH 4.8 as well as inside the range 4.8–7.0; *alka-tolerant,* above pH 7.0 as well as inside the range 4.8–7.0; and *amphi-tolerant* both below pH 4.8 and above pH 7.0, as well as within the range 4.8–7.0 (**25,** p. 129). This grouping seems to bring together, as distinctive floras, most natural groups of plants which are characteristic of special habitats, and at the same time it accounts for the presence in some such special habitats of plants which are also fairly common elsewhere.

The recognition of tolerances, which with some plants are wider than with other plants, raises the point that both wide and narrow tolerances are more or less specific or varietal characteristics of the kinds of plants concerned. This in its turn raises the question as to whether this characteristic depends upon the plant as a whole organism, or upon specific chemical characteristics of the plant or some part of the plant.

It has been suggested by SMALL (**25,** pp. 129–132) that these tolerances and narrower ranges are correlated causally with the buffer-complexes of the plants concerned. This suggestion is based upon the fact that most organic acids tend to buffer more strongly against a change of pH as acidity increases or pH decreases below pH 5, while phosphates and bicarbonates tend to buffer more strongly as the acidity decreases or pH increases from pH 5.0 to 6.5 (**24, 25**). Very few cases of plant sap with pH above 7.0 are known, and these are mainly found among marine algae and a few fungi.

This concept brings soil-pH tolerances into rational connection with the concentration and composition of the buffer-complexes inside the plant, and thus into a more or less direct relation to the metabolism of any particular kind of plant. It means that the ecological and agricultural limitations or tolerances of plants in relation to soil-pH values may be dependent upon particular characteristics of specific physiology; and it seems worth while to examine these particular characteristics with more detailed attention than has yet been given to them. The following account of a first attempt makes no claim to be complete, or widely based or conclusive; but there is enough

to make a case for more general attention and for more detailed examination of these phenomena.

Materials

Since the roots and underground parts are in closer contact with soil-fluids than are the subaerial stems and leaves of plants, it seemed to us reasonable to begin with the expressed sap of some kinds of roots, taproots, and tubers. In order to connect up at once with any possible agricultural significance, and at the same time to use plants for which soil-pH preferences are known by empirical practice and by research directed to that special point, we started with root-saps of oat, barley and wheat, and examined also taproot-sap from turnips (swedes) and tuber-sap of potatoes. The cereals were grown in garden-plots in soil with a pH of about 5.8: the turnips had been grown in medium loam of soil-pH 5.6 with available phosphate at 80 p.p.m., and the potato tubers were those of commerce. The types of materials used were Scotch Victory oats, Ayr Bounty barley, Squarehead Master wheat, Carter's 99 Semi-tankard Purple Top swede, and British Queen potato.

Methods

The saps were expressed in a Small monel-metal plant-press, and filtered immediately through an asbestos pad in a Gooch crucible. Partial analysis was begun at once on one portion of the filtrate which required no preservative methods when the first stages immediately succeeded expression of the sap. The precipitates were collected and weighed by using a pair of Schuster drop-bottles, an apparatus which was similar to the Schwartz-Bergkampf paired beakers used in microchemistry but allowed greater volumes of liquid in each of the bottles. The remainder of the sap was used for the determination of the initial pH, then a few drops of relatively strong hydrochloric acid were added and an ordinary titration was performed on the acidified fluid, using standard sodium hydroxide, 0.10 to 0.01 N, to suit the strength of sap and avoid too much dilution. In this way only one titration was required, the pH being measured on a Marconi pH-meter after each successive addition of 0.1 ml. from a micro-burette.

The titration curves were plotted on graph-paper and direct measurements from these curves were used for the calculation of buffer-index values as $\beta = \frac{d\,\mathrm{B}}{d\,\mathrm{pH}}$, where d pH = 0.5, and d B = the molar quantity of base required to raise the pH by 0.5 of a pH unit (**24, 25, 26**). From these smoothed curves, buffer-index values can be read-off for a shift of 0.2 pH unit for purposes of comparison. Curves calculated with d pH = 0.2 may be affected by the experimental error of the micro-burette manipulation and the readings from the titration-curves: where the volume of alkali needed for a given change of pH is relatively large this error should be relatively small, but for small volumes it does introduce irregularities which are experimental

in their origin and which are smoothed out by using a change over 0.5 pH units (**24**).

The phosphate estimations were made by a molybdate method (**4, 24,** p. 379); and the organic acids were estimated by the method of Hilger and Cross as developed by INGOLD and ARMSTRONG (**24,** p. 381). A special estimation of isocitric acid in potato tuber-sap was attempted, using a cucumber-seed modification of the technique described by KREBS and EGGLESTON (**11**), without using the colorimetric estimation of citric acid described by PUCHER, SHERMAN and VICKERY (**19**). The latter and other modern methods of organic acid analyses involving colorimetry and fractional distillations of esters were not available here on account of difficulties concerning apparatus and accommodation. The old methods, however, are more likely to have missed some kinds of organic acids than to have yielded too low figures for the concentrations of organic acids known to be present. For a complete analysis, as distinct from a plain titration, the facilities required are those of a chemical or biochemical rather than a botanical laboratory: even then a complete analysis may be difficult with modern technique as is shown by the 51 per cent. unknown organic acids in narcissus bulbs (**28,** p. 45).

Results

The analytical estimations include as unimportant buffer-systems in potato tuber-sap 0.0001 M oxalate and possibly 0.0002 M *iso*-citrate; in oat root-sap a trace of oxalate, in wheat root-sap 0.0001 M oxalate; and the quantities for malate, citrate, and phosphate given in table I.

TABLE I

PLANT	PART	MOLAR CONCENTRATIONS		
		MALATE	CITRATE	PHOSPHATE
Oat	Root-sap	0.0288	0.0020	0.0115
Potato	Tuber-sap	0.0046	0.0037	0.0032
Barley	Root-sap	0.0054	0.0036	0.0039
Wheat	Root-sap	0.0038	0.0009	0.0020
Turnip	Taproot-sap	Trace	Nil	0.0052

Analytical results by others include the following:

1. Oat shoots are reported (**16**) to contain citrate, malate, oxalate 0.04 per cent., also aconitic and malonic acids.

2. Barley shoots are reported (**16**) to contain citrate, malate, oxalate 0.019 per cent., also aconitic, malonic, and tricarballylic acids.

3. Wheat shoots are reported (**15**) to contain water-soluble malate, citrate, a trace of oxalate, also aconitic and malonic acids. INGOLD (**10,** table II) gave buffer-index values for shoot-sap of wheat, ranging from 0.027 for pH 4–5 to 0.013 for pH 6–7.

4. Turnip juice was examined by INGOLD (9) and yielded buffer-index values from 0.0124 for pH 4.0–4.5 to 0.0062 for pH 6.5–7.0.

5. Potato tuber-sap has been reported upon by a number of workers. The values given in table I are lower than those reported by INGOLD (**9, 10**), and still more below those of Guthrie's results (ℰ, **25**, p. 21). OSTANIN (**17**) notes that the saps of late varieties of potato give higher buffer-index values than those of early varieties. NELSON and CURL (**13**) agree with Guthrie in general but report the presence of some oxalate (**9, 10**) and some iso-citric acid.

The quantities of main buffer-systems given in table I account for various proportions of the buffering capacities of the sap; the proportion explained varying with the sap and the pH at which it is calculated. For example, a mere trace of possible malate was the only organic acid identified for turnip taproot-sap, and the phosphate at 0.0052 M accounts for 100 per cent. of the buffer-index at pH 6.4–6.8, but for none of the buffer-index (0.0038) at pH 4.4–4.6. Similarly the known malate-citrate-phosphate component of the buffer-complex in root-sap of oats accounts for only about 47 per cent. of the buffer-index at pH 4.6–4.8, but 79 per cent. at the initial pH 6.1, 87 per cent. around pH 6.5 and 98 per cent. around pH 6.7. In general the phosphate part of the buffering is completely known, while deficiencies are still present in our knowledge of the organic acid part of these buffer-complexes.

The total buffer capacity varies throughout the pH scale, and the part of that scale which is most important within the plant lies between pH 4.0 and pH 7.0. The critical points in relation to soil-pH values and tolerances have been taken as pH 4.6–4.8, the initial pH, and pH 6.4–6.6. The total molar concentration of organic acids in the root-sap of oats is about 0.066 M (of which the malate and citrate found account for 0.0308 M), and this may be compared with about 0.05 M for apple juice which is mainly malate.

Taking the highest buffer-index in the present data as a standard at 100, for root-sap of oats at pH 4.6–4.8 ($\beta = 0.0423 = 100$), we can more readily compare the other buffer-index values within this set of data. The possibility of comparisons for all buffer-index values is one of the main reasons why Van Slyke's $d\ B/d$ pH method of presenting these indices should be regarded as "standard," and curves for all five plant saps have been provided with this account. These are published here as figure 1, and contain all the essentials for comparisons with other data within the limits pH 4.0 to pH 7.0. Table II presents a selection of the data for particular pH zones, as percentages of the highest value around pH 4.7 (0.0423).

Most varieties of oats are acid-tolerant, and ARRHENIUS (**3**) gave records of alka-tolerance for several of his experimental varieties, so that the Scotch Victory variety may be classed provisionally as amphi-tolerant, with buffer-index values 0.0423 at pH 4.6–4.8, and 0.0108 at pH 6.4–6.6. Apart from the increased incidence of several diseases above pH 6.0, the potato may also be classed as amphi-tolerant, with buffer-index values 0.0199 at

pH 4.6–4.8, and 0.0070 at pH 6.4–6.6. Barley is definitely not acid-tolerant, with buffer-index below 0.02 (0.0144) at pH 4.6–4.8, but it is usually alka-tolerant and Ayr Bounty root-sap had a buffer-index of 0.0042 around pH 6.5.

Wheat has alka-tolerant varieties, but ARRHENIUS (3) records one variety as showing an optimum about soil-pH 5.3 and little or no pH tolera-

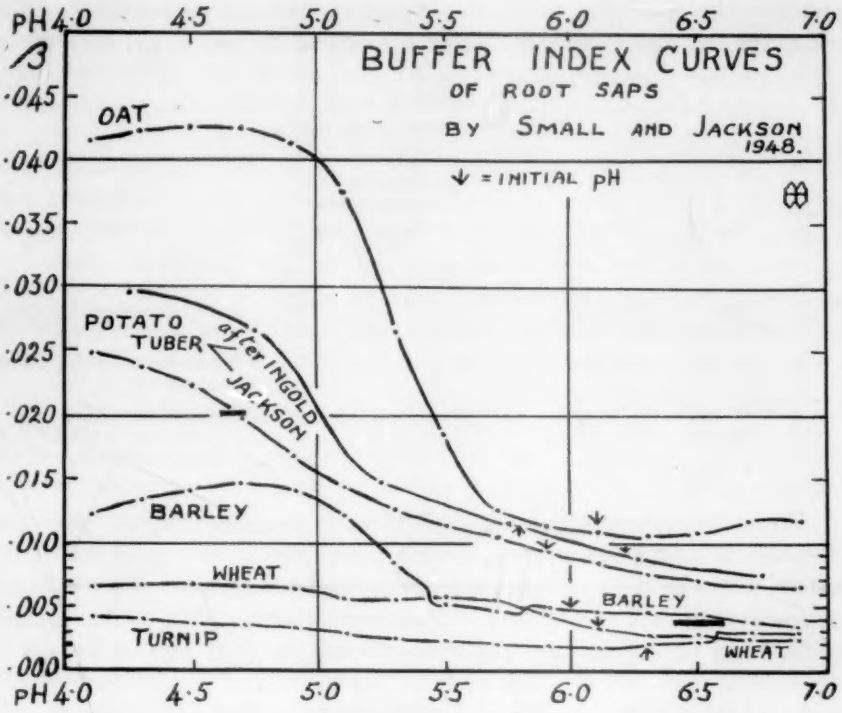

FIG. 1. Buffer index curves of root saps.

tion on either side of that optimum, so the Squarehead Master variety may be of this mesophilous type with buffer-index values at both extremes below the minimal values for tolerances: 0.0065 at pH 4.7 and 0.0026 at pH 6.5 for this wheat root-sap; the minima being about 0.02 for acid-tolerance and about 0.004 for alka-tolerance. Rye grows over a wide range of soil-pH, but grows well only between pH 5.0 and pH 7.0: the root-sap has not yet been examined, but on the above data for cereals the buffer-index should be about 0.02–0.03 around pH 4.7 and probably little more than 0.004 around pH 6.5.

Turnips are recognized as growing well only within the upper mesophilous range pH 6.0–7.0, while swedes grow best in the lower mesophilous range pH 5.0–6.0. This is a reasonable correlation with buffer-index values for taproot-sap 0.0037 around pH 4.7 and 0.0025 around pH 6.5, both

below the apparent minimal values for tolerances below or above the mesophilous range.

These lower limits, of 0.02 around pH 4.7 and 0.004 around pH 6.5 in root-saps, for acid-tolerance and alka-tolerance respectively are indicated by the limited data in table II. They are not conclusively proved deductions, but their agreement with previously suggested correlations are considered to be sufficient to encourage other attempts, on a wider scale, to investigate the possibility of ecological and agricultural correlations of soil-pH tolerances and total buffer-index values over the range pH 4.0–7.0.

TABLE II

RELATIVE BUFFER INDEX VALUES

INITIAL pH	PLANT	PART	RELATIVE BUFFER INDEX VALUE			TOLERANCES
			pH 4.6–4.8	Initial pH	pH 6.4–6.6	
6.1	Oat	Root-sap	100	26	26	Acid-tolerant with β 0.02 or more at pH 4.7
5.9	Potato	Tuber-sap	47	22	17	
6.0	Barley	Root-sap	34	11	10	Alka-tolerant with β 0.004 at pH 6.5
6.1	Wheat	Root-sap	15	7	6	Mesophilous with β less than 0.02 at pH 4.7 and less than 0.004 at pH 6.5
6.3	Turnip	Taproot-sap	9	5	6	

From the physiological point of view, it is interesting to note that much of the buffering capacity of these plants can be accounted for by the known concentrations of malates, citrates, and phosphates. This is in accordance with data for other parts of many plants (**25**). Other organic acids found to be important buffer-components in other plants, but not in the present five, are oxalates and tartrates (**7, 8**). These four organic acids, together with phosphates, account more or less completely for the buffer-complex of sap expressed from young stems of *Pelargonium zonale,* (**2**) and SMALL (**25**, p. 19). As an example of a simpler buffer-complex, malate and phosphate in molar concentrations such as were found by Thoday and his collaborators in *Kleinia articulata* account for all the buffer-index values calculated from their titration data between pH 4.75 and pH 6.75, both sets of data varying from young plants to old plants (see **25**, p. 171). ARMSTRONG (**1**) also found that the oxalate-citrate-phosphate content accounted completely for the buffer-index curve of sap from the fructifications of the fungus *Coprinus micaceus,* while for *Collybia velutipes* the complete buffer-complex consisted of malate-citrate-phosphate.

The general occurrence of these four organic acids, sometimes in concentrations sufficient to account for all the buffering capacity within the normal living zone (pH 4.0–7.0), gives a special interest to any cases where they do not explain all the buffering, particularly where most of the acid-buffering seems to be due to other components. Among these other

components may be succinates (**20**), *iso*-citrates (**8, 11, 12, 13, 14, 21**), aconitates (**11, 16**) malonates, and salts of tricarballylic acid (**16**), possibly amino-acids (**9, 10**), also acetates and lactates (**24, 25**). The buffering effects of the carbonic-bicarbonate system are probably quite important in cells with sap at pH 5.0–6.5, but most of the carbonic acid is normally removed during the processes of expression and filtration, so this buffer system is unlikely to be important in these expressed juices.

The analytical methods used for this investigation should indicate and estimate succinates and lactates when these are present in any significant concentration, as well as the usual four—malate, citrates, oxalates, tartrates. The accuracy and efficiency of the analytical methods used were checked against known mixtures, and also against cherry fruit sap where both the shape and very nearly all the values along the buffer-index curve were accounted for by the components as estimated at—malate 0.0043 M, citrate 0.0016 M, and phosphate 0.0036 M, which added together along their own curves explained 81 to 100 per cent. of the curve for the sap. Other methods for organic acid analyses have been devised, and our knowledge of the organic acids in plants has increased considerably since 1929 (**24, 6, 7, 8, 11, 12–16, 18–23, 27–28**).

Comparable buffer index values

The titration, using a pH-meter, of an acidified expressed sap from roots or other underground parts of plants is a comparatively simple operation, and the accurate graphing of a titration curve should give no serious trouble. The subsequent calculation and graphing of a buffer-index curve as d B/d pH is only one step further. The graph or tabulation of buffer-index values over the range pH 4.0–7.0 yields results which can be compared over a wide variety of plants and their parts, also with similar results obtained by other workers anywhere.

Since the buffer-index varies arithmetically as the molar concentration, since total buffer-index values are simply additive for all components at any one given pH, and since the shapes of buffer-index curves along a basal pH scale are characteristic for each acid and its salts, buffer-index curves are very useful checks on analyses, and present the total picture of the buffer-capacity of known and unknown substances in the titrated material.

If the generalizations concerning the causal correlation of root-sap buffer-index values around pH 4.7 and pH 6.5 with acid-tolerance, alka-tolerance, and amphi-tolerance, also with mesophilous characteristics, as shown by naturally growing or agriculturally cultivated species and varieties, if all or any of this be true, then ecological preferences for limited ranges or ecological tolerances over wider ranges of soil-pH should be capable of a specific chemical explanation. Further, extensive field trials of agricultural soil-pH tolerances could be either preceded or replaced provisionally by a straight determination of the buffer-index curve of root-sap

from a small sample. It might even be possible to make the liming of soils more economically and rationally controlled by consideration of buffer-index curves for both the soil and the roots of proposed crops.

Summary

1. Some data are given for the relative buffer-index values of saps from underground parts of wheat, barley, oat, potato, and turnip.

2. Partial analyses of these saps are given, and the citrate-malate-phosphate components of the buffer-complexes are found to explain a large proportion of the buffer-index values.

3. It is suggested that the minimal buffer-index values of root-saps are 0.02 around pH 4.7 for acid-tolerance and 0.004 around pH 6.5 for alka-tolerance of plants in relation to soil-pH values below pH 4.8 and above pH 7.0 respectively.

4. Some possible practical applications for data of this type are indicated.

Department of Botany
The Queen's University of Belfast
Northern Ireland

LITERATURE CITED

1. Armstrong, J. I. Hydrion concentration and buffers in fungi. Protoplasma **8**: 222–260. 1929.
2. ————. Buffer-complex of sap from stems of Pelargonium. Protoplasma **8**: 313–343. 1929.
3. Arrhenius, O. Kalkfrage, Bodenreaktion und Pflanzenwachstum. Leipzig. 1926.
4. Embden, G. Eine gravimetrischen Bestimmungsmethode für kleine Phosphorsäuremengen. Z. physiol. Chem. **113**: 138–145. 1921.
5. Guthrie, J. D. Metabolism of citric, sulphuric, and nitric acid in the potato tuber. Cont. Boyce Thompson Inst. **6**: 247–268. 1934.
6. Hartman, B. G., and Hillig, F. Determination of citric acid in fruit and fruit products. Jour. Assoc. Official Agric. Chem. **13**: 99. 1930.
7. ————. Determination of tartaric acid in fruit and fruit products. Jour. Assoc. Official Agric. Chem. **13**: 103. 1930.
8. ————. Determination of polybasic acids of fruits and fruit products. Jour. Assoc. Official Agric. Chem. **26**: 444. 1943. See also "Methods of Analysis, Official and Tentative" 6th edit. 1945, published by the Association of Official Agricultural Chemists.
9. Ingold, C. T. Buffers of potato, tuber and leaf. Protoplasma **9**: 441–6. 1930.
10. ————. Buffer systems of plant juices. Protoplasma **9**: 447–455. 1930.
11. Krebs, H. A., and Eggleston, L. V. Micro-determination of *iso*-citric

and *cis*-aconitic acids in biological material. Biochem. Jour. **36**: 426–437. 1944.

12. Nelson, S. K. Isocitric acid. J. Amer. Chem. Soc. **52**: 2928–2933. 1930.
13. ————, and Curl, A. L. The non-volatile acids of the potato. Amer. Potato Journal **17**: 328. 1940.
14. ————, and ————. The occurrence of citric and isocitric acids in blackberries. Jour. Agric. Res. **67**: 301. 1943.
15. ————, and Hasselbring, H. Some organic acids of the wheat plant. Jour. Amer. Chem. Soc. **53**: 1040–3. 1931.
16. ————, and Mottern, H. H. Some organic acids in barley, maize, oats and rye plants. Jour. Amer. Chem. Soc. **53**: 3046–8. 1931.
17. Ostanin, S. N. The acidic properties of the juice from potato tubers. Bull. App. Bot. Gen. Plant Breeding. Ser. III. **14**: 159. 1936.
18. Pucher, G. W., Vickery, H. B., and Leavenworth, C. Determination of the acids of plant tissues. III. Citric acid. Ind. Eng. Chem. Anal. Ed. **6**: 190–2. 1934.
19. ————, ————, and Sherman, C. C. The determination of citric acid in biological material. Jour. Biol. Chem. **113**: 235–245. 1946.
20. ————, and ————. Succinic acid as a metabolite in plant tissues. Plant Physiology **16**: 771–784. 1941.
21. ————, and ————. Identity of the so-called Crassulacean malic acid with isocitric acid. Jour. Biol. Chem. **145**: 525–532. 1942.
22. ————, and Wakeman, A. J. Determination of malic acid in plant tissue. Ind. Eng. Chem. Anal. Ed. **6**: 288–291. 1934.
23. ————, and ————. Organic acids in plant tissues. Ind. Eng. Chem. Anal. Ed. **13**: 244–246. 1941.
24. Small, J. Hydrogen-ion Concentration in Plant Cells and Tissues. Berlin. 1929.
25. ————. pH and Plants. London and New York. 1946.
26. Van Slyke, D. D. On the measurement of buffer values and on the relationship of buffer value to the dissociation constant of the buffer, and the concentration and reaction of the buffer solution. Jour. Biol. Chem. **52**: 525. 1922.
27. Vickery, H. B., and Pucher, G. W. Organic Acids of Plants. Ann. Rev. Biochem. **9**: 528. 1933.
28. ————, ————, Wakeman, A. J., and Leavenworth, C. S. Chemical investigations of the metabolism of plants. I. The nitrogen nutrition of *Narcissus poeticus*. Connecticut Agric. Expt. Sta. Bull. **496**. 1946.

OBSERVATIONS ON THE MECHANISM OF AUXIN FORMATION IN PLANT TISSUES

S. G. Wildman and R. M. Muir

(WITH TWO FIGURES)

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Auxin physiologists often have attempted to establish correlations between the amount of auxin in a plant tissue and the manifestation of auxin activity such as growth, rooting, parthenocarpy, bud inhibition, etc. With Avena coleoptiles and tomato stem tips, to use specific examples, it is possible to demonstrate a correlation between growth and the amount of auxin which can be diffused from the tissue into agar. Many tissues produce too little diffusible auxin or are too cumbersome to lend themselves to this type of auxin analysis and resort must be made to more convenient methods of auxin extraction. Ether extraction has been used extensively in correlation studies, particularly since ether was considered to remove the free auxin. It soon became evident, however, that auxin was liberated during the extraction and that many months of successive ether extractions were required before auxin production disappeared. While there often appeared to be a correlation between the amount of auxin obtained in the first ether extraction and the growth process under consideration, the correlation usually disappeared as the extraction process was prolonged.

Thimann and Skoog (**8**) made an extensive study of the continued production of auxin during the extraction of fresh, green tissues with ether and came to the following conclusions: 1) Many successive ether extractions spaced over several months were required to extract all of the potentially ether soluble auxin contained in Lemna tissue. 2) Water was necessary for auxin production during ether extraction since oven-dried tissue failed to give off auxin when extracted with anhydrous ether. 3) Drying not only prevented auxin production but appeared to fix added auxin in some form not extractable with ether. 4) Addition of proteolytic enzymes to the tissue prior to ether extraction greatly increased the total amount of auxin which could be extracted but did not shorten the time appreciably nor the number of extractions required for total auxin extraction. They concluded that the continued production of auxin in ether was caused by the slow, enzymatic liberation of auxin from an inactive form, presumably a protein.

Gustafson (**4**) has developed a method for the extraction of "free" auxin, or auxin which is present in the tissue as such but does not arise by liberation during the extraction process. The tissue is frozen, ground and then boiled with water before extraction with ether. Boiling prevents the continued production of auxin encountered in previous methods. While

the method has proven quite satisfactory for some tissues, in others boiling evidently liberates some material which inhibits the action of auxin in the Avena test (**9**).

Recent work has shown that spinach leaves contain an enzyme which rapidly converts tryptophan to auxin (**10**). Because of the existence of such an enzyme, it is possible that one of the reasons for the slow production of auxin during ether extraction is the failure to inactivate this enzyme prior to extraction. The present investigation is therefore an attempt to associate the auxin production process with some of the known properties of the tryptophan-auxin converting enzymes and to examine the possibility of inactivating the enzymes by less drastic means than used previously thus enabling one to measure "free" auxin with confidence. Fertilized tobacco ovaries are advantageous for this kind of study because large amounts of auxin can be diffused into agar from them (**6**) and thus free auxin and diffusible auxin can be compared. Such tissues can be obtained in reasonably large quantities in contrast to other tissue such as Avena or corn coleoptiles from which auxin can be obtained by diffusion.

Methods and materials

PREPARATION OF PLANT MATERIALS.—Since the principal aim of this work was to investigate the nature of free auxin and its origin in plant tissues, it was necessary that the free auxin should be immobilized completely at the time of sampling and maintained in an immobilized condition during subsequent extraction procedures. Hence, fertilized tobacco ovaries were dissected from the flowers 80–100 hours after pollination and plunged immediately into liquid air for rapid freezing. They were then dried *in vacuo* in the frozen state in a Campbell-Pressman lyophil apparatus (**2**), a method closely similar to that used by LINK, EGGERS and MOULTON (**5**) for the preparation of plant material for auxin analysis. When dry, the ovaries were ground to 40 mesh in a Wiley mill and then stored *in vacuo* over P_2O_5 in darkness until used as tissue for auxin analysis or as enzyme preparations.

ETHER EXTRACTION.—Weighed samples of dried ovarian tissue were placed in 150-ml. Erlenmeyer flasks and covered with 75 ml. of freshly-distilled, peroxide-free ether, unless otherwise indicated. Such ether has a water content of about 5%. The samples were occasionally shaken. At the end of the extraction period the ether was decanted from the plant tissue, the sample was washed twice with small portions of fresh ether, and the combined ether extracts were reduced in volume to a few milliliters which were then quantitatively transferred to melted agar which was used for Avena assay.

AVENA DETERMINATION.—The methods used in this investigation were exactly as described previously (**10**). Auxin was applied to individual oat coleoptiles in 8.76 $mm.^3$ agar blocks. Results compared on the basis of curvatures were either obtained from the same Avena experiment or the necessary sensitivity values are given.

Experimental results

DIFFUSION AND EXTRACTION OF AUXIN FROM FERTILIZED TOBACCO OVARIES.—As previously demonstrated (**6**) large quantities of auxin can be diffused out of fertilized tobacco ovaries into agar. Eighty-five hours after pollination, twelve ovaries were excised from the flowers and the basal cut surfaces were placed on 8.76 mm.^{3} agar blocks for four hours. When these blocks were tested for auxin activity, an average of 21.2° curvature was produced as shown in table I. The amount of diffusible auxin was compared with the

TABLE I

COMPARISON OF THE AMOUNTS OF AUXIN OBTAINED FROM TOBACCO OVARIES BY VARIOUS METHODS

METHOD	MG. DRY WEIGHT	NO. OF OVARIES	CURVATURE	ML. OF AGAR	SENSITIVITY 50 γ IAA/LITER	TOTAL CURVATURE PER OVARY
Fresh ovary diffused on agar for 4 hours	4	1	21.2 ± 1.8	8.76×10^{-3}	11.8	21.2
Lyophilized tissue extracted 1 hour at 23° C. with dry ether	20	5	0.0	0.4	11.8	<5.0*
Lyophilized tissue extracted 1 hour at 0° C. with wet ether	20	5	0.0	0.4	11.8	<5.0*
Lyophilized tissue extracted 1 hour at 23° C. with wet ether	20	5	6.3 ± 0.6	0.4	14.3	47.6
Lyophilized tissue extracted 12 hours at 23° C. with wet ether	20	5	17.8 ± 1.7	0.4	10.6	181
Lyophilized tissue incubated 3 hours with 1.0 mg. of tryptophan	10	2.5	9.4 ± 1.0	3.2	8.3	1953

* Minimum sensitivity of Avena test.

amount of auxin which could be extracted from the tissue by different ether treatments. The free auxin was immobilized by freezing in liquid air and the tissue was dried in the lyophil apparatus before ether extraction. As the data in table I indicate, no auxin was obtained from five ovaries by extraction, either with anhydrous ether for one hour at 23° C. or with freshly-distilled, "wet" ether when the tissue and solvent were kept at 0° C. for one hour. If the tissue and solvent with water were maintained at 23° C. however, auxin causing 6.3° curvature was obtained in one hour, and in 12 hours the auxin obtained was sufficient to produce 17.8° curvature. When the total amounts of auxin obtained per ovary were compared, it was found that the amount of auxin extracted in one hour at 23° C. actually exceeded the amount obtained by diffusion from the fresh ovary in four hours.

On the basis of the above data, it is evident that rapid auxin production occurs during ether extraction of lyophilized tobacco ovaries provided the

tissue is rewetted and a suitable temperature of extraction is used. The fact that the production of auxin could be prevented completely by keeping the tissue and solvent cold or by keeping them dry suggests at once that the process is enzymatic. It was of interest therefore to see if the production of auxin could be increased in the presence of excess tryptophan. That this is true is shown in table I. Lyophilized tobacco ovary tissue was incubated for three hours at 28° C. with 1.0 mg. of tryptophan in 3.0 ml. of phosphate buffer, pH 7.0. At the end of this period the digest was centrifuged free of cellular constituents, the supernatant liquid was acidified, and the auxin was extracted with ether (10). When tested by Avena assay, curvature equivalent to 1953° per ovary was obtained. Other experiments not presented here

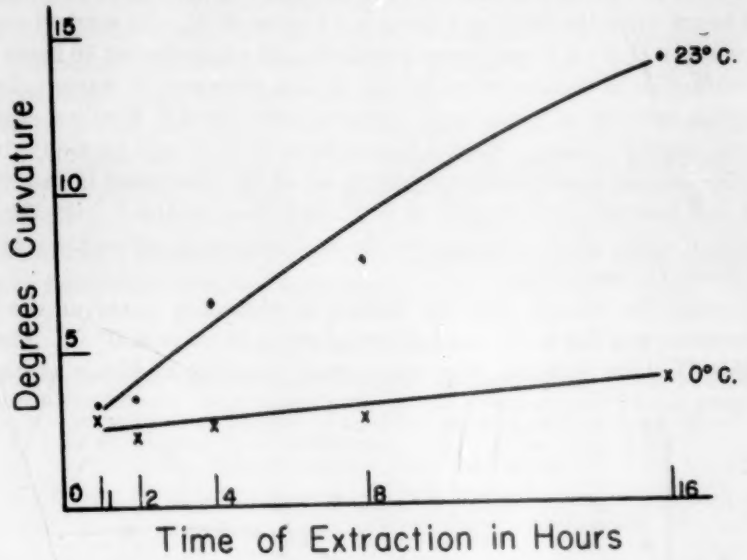

FIG. 1. The amount of auxin produced by wet ether extraction as effected by time and temperature of extraction. Twenty mg. samples of lyophilized tobacco ovaries.

have shown that boiling the tissue prior to adding tryptophan prevents the production of auxin. It can be concluded that tobacco ovaries contain a very active tryptophan-auxin converting enzyme which is not destroyed by the lyophilization procedure.

Because of the short time intervals involved in all of these experiments, it is exceedingly doubtful that the hydrolysis of auxin-protein complexes could account for the auxin produced. As a matter of fact, very drastic treatment such as heating for prolonged periods in the presence of alkali, or long incubation with proteolytic enzymes is necessary to release auxin from its bound form in spinach leaves (11). Another explanation of rapid auxin production must be considered and the following experiments have been designed to show that auxin production during ether extraction is a consequence of the enzymatic conversion of tryptophan to auxin.

TABLE II

RECOVERY OF AUXIN ADDED TO 10-MG. SAMPLES OF LYOPHILIZED TOBACCO OVARY TISSUE BY EXTRACTION WITH WET ETHER AT 0° C.

PERIOD OF EXTRACTION AT 0° C.	IAA ADDED IN DEGREES CURVATURE	IAA RECOVERED IN DEGREES CURVATURE	RECOVERY %
2 hours	5.1 ± 0.9	5.1 ± 1.0	100
3 hours	12.4 ± 1.1	10.3 ± 0.9	83

THE EFFECT OF TEMPERATURE ON THE EXTRACTION OF AUXIN.—The data presented graphically in figure 1 show that the production of auxin in lyophilized tobacco ovaries can be reduced to a very low value for periods up to 16 hours when the ether and tissue are kept at 0° C. In marked contrast to this result there is a continuous production of auxin during 16 hours when the extraction is conducted at 23° C. in the presence of water. Twenty milligram samples of tissue were extracted with freshly distilled ether for various lengths of time. Since a temperature of 0° C. will partially dry the ether by causing some water to separate, all of the ether used in this experiment was maintained overnight at 0° C. and then decanted from the small amount of water which separated by the cold treatment before the ether was used for auxin extraction.

It might be thought that the failure in obtaining auxin at the lower temperature was due to the insolubility of auxin in ether at 0° C. The data in table II show, however, that when small amounts of indole acetic acid

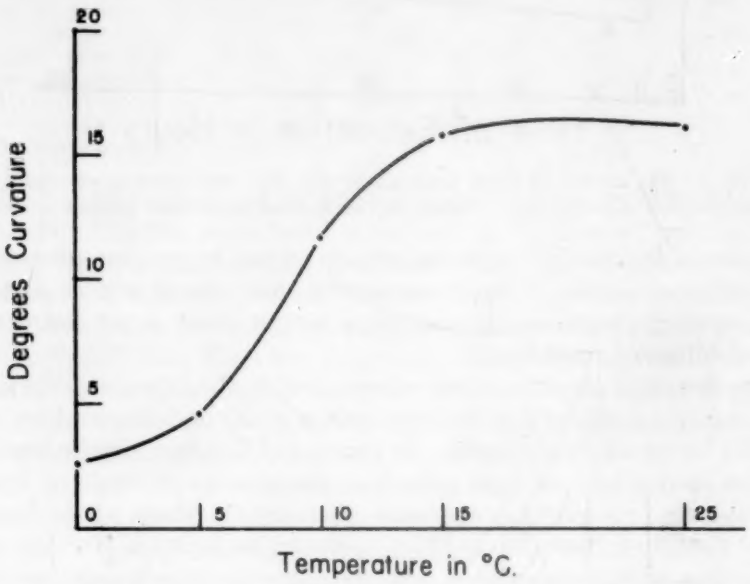

FIG. 2. The amount of auxin produced by wet ether extraction at different temperatures. Twenty mg. samples of lyophilized tobacco ovaries extracted 16 hours.

(IAA) were added to the tobacco tissue prior to extraction with ether at 0° C., the added auxin was quantitatively recovered by ether extraction.

The data plotted in figure 2 confirm the enzymatic nature of the auxin production process. Samples of tobacco ovary tissue weighing 20 mg. were extracted with ether for 16 hours at 0, 5, 10, 15 and 25° C. When the amount of auxin produced is plotted against temperature of extraction, the resulting curve approximates the data for typical enzymatic processes (**7**). For each 5° rise in temperature to 15° C. the amount of auxin produced is nearly doubled. At 15° C. an optimum production is attained. The low boiling point of the ether prevented an extension of this curve to higher temperatures at which it might be anticipated the auxin production would fall due to heat inactivation of the enzyme.

As a consequence of these temperature relationships it is possible to conclude that auxin production during ether extraction of lyophilized tissues resembles an enzymatically activated process.

THE EFFECT OF CYANIDE ON THE FORMATION OF AUXIN DURING ETHER EXTRACTION.—Small amounts of cyanide completely block the enzymatic conversion of tryptophan to auxin by spinach leaf breis (**10**). Since tobacco ovaries were also found to contain an active tryptophan-auxin converting enzyme, it was of interest to see if the production of auxin could be blocked when the tissue was extracted with ether in the presence of cyanide. There

TABLE III

EFFECT OF CYANIDE ON THE FORMATION OF AUXIN IN WET ETHER. 20-MG. SAMPLES OF LYOPHILIZED TOBACCO OVARY TISSUE EXTRACTED 16 HOURS AT 23° C. WITH ETHER IN THE PRESENCE OR ABSENCE OF CYANIDE. SODIUM CYANIDE DISSOLVED IN WATER AT SUCH A CONCENTRATION THAT 0.5 ML. WAS ADDED IMMEDIATELY BEFORE ETHER EXTRACTION

	Mg. NaCN added	IAA added in degrees curvature	Total auxin extracted in degrees curvature	Apparent % inhibition of auxin production	% inhibition of the Avena assay
Composite #1	0.0	0	16.0 ± 1.4	0	.....
" "	1.0	0	3.5 ± 0.6	78	.....
" "	1.0	17.5 ± 1.3	11.0 ± 1.2	.....	57
Composite #2	0.0	0	9.5 ± 0.8	0	.....
" "	0.5	0	4.2 ± 0.5	56	.....
" "	0.5	12.9 ± 0.8	11.8 ± 1.1	.....	41
" "	0.25	0	4.8 ± 0.7	50	.....
" "	0.25	12.9 ± 0.8	15.8 ± 1.4	.....	15

are certain difficulties attached to the use of cyanide in this type of experiment which need to be mentioned. Both HCN and NaCN are soluble in ether. While it is a simple matter to get rid of HCN by evaporation since it boils at a temperature below the boiling point of ether, this is not the case with NaCN and there is always the danger that this poison will be carried over into the agar used for the Avena test and thus prevent the coleoptiles from reacting to the auxin. The pH of the plant tissues rewetted with water

from the ether is about 6.3 and it is to be expected that both HCN and NaCN will be present in the ether extracts at this hydrogen-ion concentration. To ensure that a reduction in auxin production was a result of inactivation of the enzyme rather than the inhibition of the Avena assay, IAA was added in the presence of cyanide to all of the tissues involved so that it is possible to estimate the degree of inhibition of the enzymatic production of auxin during ether extraction.

Two different lots of lyophilized tobacco ovaries were used in these experiments. Samples of tissue weighing 20 mg. each were extracted for 16 hours at 23° C. The data are presented in table III. The auxin obtained from the tissue in the absence of cyanide produced 16.0° curvature. In the presence of 1.0 mg. of NaCN auxin production was reduced to 3.5° curvature. Since the apparent auxin production was inhibited 78% by this amount of

TABLE IV

EXTRACTION OF AUXIN FROM VARIOUS PLANT TISSUES BY ETHER. EXTRACTION PERIOD OF 16 HOURS

TISSUE	MG. DRY WEIGHT	TEMPERATURE DEGREES C.	ML. OF AGAR	CURVATURE
Avena coleoptile tips	10	0	0.25	7.4 ± 0.5
" " "	10	23	0.25	13.2 ± 0.9
Tomato stem tips	20	0	0.25	3.2 ± 0.7
" " "	20	23	0.25	9.2 ± 0.8
Carrot root	20	0	0.30	0
" "	20	23	0.30	2.5 ± 0.8
Corn coleoptile tips	10	0	0.30	6.3 ± 0.3
" " "	10	23	0.30	8.8 ± 0.6

cyanide and the Avena assay was inhibited only 57%, it is evident that an enzymatic blocking or inactivation had occurred in the auxin production process. When the experiment was repeated with a second lot of ovaries and smaller amounts of cyanide it was more evident that the cyanide prevented the formation of auxin. The extraction of 20 mg. of tissue without cyanide yielded sufficient auxin to produce 9.5° curvature but in the presence of 0.5 mg. or 0.25 mg. of NaCN the yield of auxin was reduced 50%. With the higher concentration of cyanide there was 41% inhibition of the Avena assay but with the lower concentration the inhibition was only 15%. The effect of cyanide on the production of auxin during ether extraction bears a striking resemblance to the effect of cyanide on the tryptophan-auxin converting enzyme of spinach leaves. This fact, coupled with the rapidity of the formation process as a function of the temperature of extraction, strongly suggests that auxin production results from the enzymatic conversion of tryptophan.

ETHER EXTRACTION OF OTHER LYOPHILIZED PLANT TISSUES.—That rapid auxin production during ether extraction is not limited to tobacco ovaries is shown by the data in table IV. Etiolated corn and Avena coleoptile tip

tissue, green tomato stem tip tissue, and carrot root tissue were prepared in exactly the same way as the tobacco ovary tissue. Although the levels of free auxin, as indicated by the 0° C. extraction, were much higher for the etiolated tissues, in all cases more auxin was produced in a 16-hour ether extraction period at 23° C. than in an identical period at 0° C. It is probable that the same mechanism operative in tobacco ovaries also accounts for the production of auxin in the other tissues.

Discussion

On the basis of these experiments which show that rapid auxin production in lyophilized tissues is probably due to the enzymatic conversion of tryptophan to auxin, it is possible to suggest a method for the determination of free auxin with confidence that this is actually the auxin state being determined. Obviously, the tryptophan-auxin enzyme must be inactivated at the time of sampling and kept inactivated during the extraction with ether. The preferable method is rapid freezing of the tissue followed by drying in the lyophil apparatus and ether extraction conducted at 0° C. for periods up to 16 hours. This treatment causes the least possible change in the tissue. Boiling the tissue in order to inactivate the enzyme is unsatisfactory because of the danger of liberating inhibitors of the Avena test (**9**) and, as will be shown in a future publication, at least 15 minutes at 100° C. is necessary to inactivate completely the tryptophan-auxin converting enzyme in Avena coleoptiles.

These results help to explain the difficulties encountered previously in auxin extraction, and also substantiate a previous hypothesis (**10**) that tryptophan is a principal precursor of auxin in plants. There is no experimental evidence which suggests that plants contain enzymes capable of liberating auxin rapidly from the various bound-auxin complexes so far investigated (**1, 11**). Free tryptophan in the presence of the tryptophan-auxin converting enzyme now demonstrated in many diverse plant species, is converted to auxin, and in this sense the tryptophan-converting enzyme must necessarily be responsible for the formation of auxin.

Summary

1. Lyophilized tobacco ovaries produce auxin during ether extraction in the presence of water at 23° C. but not at 0° C. The process is shown to be enzymatic.

2. Tobacco ovaries contain an active tryptophan-auxin converting enzyme. Production of auxin during ether extraction resembles the enzymatic conversion of tryptophan to auxin as both processes are blocked by cyanide.

3. Several other plant tissues appear to produce auxin by the same mechanism found in tobacco ovaries.

4. A simple method for the extraction of free auxin is proposed.

5. The evidence presented supports the view that tryptophan is a principal precursor of auxin in plants.

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KERCKHOFF LABORATORIES OF BIOLOGY
CALIFORNIA INSTITUTE OF TECHNOLOGY
PASADENA, CALIFORNIA
AND
BOTANY DEPARTMENT
STATE UNIVERSITY OF IOWA
IOWA CITY, IOWA

LITERATURE CITED

1. BERGER, J., and AVERY, G. S. Isolation of an auxin precursor and an auxin (indole acetic acid) from maize. Amer. Jour. Bot. **31**: 199–203. 1944.
2. CAMPBELL, D. H., and PRESSMAN, D. A simplified lyophil apparatus. Science **99**: 285–286. 1944.
3. GORDON, S. A. Auxin-protein complexes of the wheat grain. Amer. Jour. Bot. **33**: 160–169. 1946.
4. GUSTAFSON, F. G. The extraction of growth hormones from plants. Amer. Jour. Bot. **28**: 947–951. 1941.
5. LINK, G. K. K., EGGERS, V., and MOULTON, J. E. Use of frozen vacuum-dried material in auxin and other chemical analyses of plant organs: its extraction with dry ether. Bot. Gaz. **102**: 590–601. 1941.
6. MUIR, R. M. Growth hormones as related to the setting and development of fruit in *Nicotiana tabacum*. Amer. Jour. Bot. **29**: 716–720. 1942.
7. SUMNER, J. B., and SOMERS, G. F. Chemistry and Methods of Enzymes. Academic Press. New York. 1943.
8. THIMANN, K. V., and SKOOG, F. The extraction of auxin from plant tissues. Amer Jour. Bot. **27**: 951–960. 1940.
9. VAN OVERBEEK, J., OLIVO, G., and SANTIAGO DE VAZQUEZ, E. A rapid extraction method for free auxin and its application in geotropic reactions of bean seedlings and sugar-cane nodes. Bot. Gaz. **106**: 440–451. 1945.
10. WILDMAN, S., FERRI, M., and BONNER, J. The enzymatic conversion of tryptophan to auxin by spinach leaves. Arch. Biochem. **13**: 131–144. 1947.
11. WILDMAN, S., and BONNER, J. The proteins of green leaves. I. Isolation, enzymatic properties and auxin content of spinach cytoplasmic proteins. Arch. Biochem. **14**: 381–413. 1947.

SOME FACTORS AFFECTING GERMINATION OF CELERY SEED

CARL A. TAYLOR

(WITH SIX FIGURES)

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Introduction

Some of the germination characteristics of celery seed are of practical importance because they dictate cultural practices which are expensive and more exacting than are required for many other crops. Better understanding of the factors affecting the rate of germination should lead to possible treatments which shorten the pre-emergence period and simplify cultural requirements.

Light, alternating temperatures, moisture, etc., are known to affect the germination of the seed, **1, 2, 3, 8,** but in these studies the interactions of the several factors and their effect on the rate of germination were not investigated or reported.

In the case of kok-saghyz and guayule seed, the difficulties due to slow germination were overcome by presprouting **4, 5**. In this paper are given the results of studies showing the influence of some factors on the rate of germination of celery seed, and their applicability for presprouting the seed prior to direct field seeding.

The germination behavior of other species of seeds has been adequately reviewed and a comprehensive review of such literature will not be attempted in this paper dealing exclusively with celery.

Materials and Methods

The term rate of germination as used in this paper refers to the time lapse between wetting or sowing of the seed and the appearance of the radicle in the case of blotter germinations or emergence of the seedling in sowing tests, the measure being the percentage germinated or emerged on successive days after wetting or sowing the seed.

Except where specified otherwise, the celery seed used was of variety Tall Utah grown by Ferry Morse Seed Company. Sodium hypochlorite solutions were made by diluting the household grade products such as Chlorox, Purex or White Magic proportionally to their rated strength in available chlorine and verifying the concentration by titration, **9**.

Laboratory germinations were run by placing replicate 100-seed samples on blotter or filter paper in Petri dishes. Where controlled temperatures are indicated they were obtained by holding the Petri dishes in thermostatically controlled germinating ovens with or without daylight. Germination

or presprouting in the dark was obtained by holding the seed containers in a lightproof ventilated metal box within the germinator or refrigerator. Except where specified otherwise, the cited alternating temperatures were at the lower temperature for 16 hours and at the higher temperature for eight hours daily.

Experimental Results

LABORATORY—When soaked celery seeds were held at 70–75° F. for a few days, they became moldy and the resulting germination was poor. Also it was noted that soaking celery seed in water extracts from the seed resulted in a reduction in germination and malformation of the seedlings. (fig. 1).

FIG. 1. Effect of seed solutes on germination of celery seed. Left: Germination on blotter moistened with water. Right: Germination on blotter moistened with strong solution of substances dissolved from celery seed. Both lots germinated 10 days at room temperature in light.

These responses have been noted in other seeds 7 and have been eliminated by treating with certain sterilizing agents that are also strong oxidants, such as sodium or calcium hypochlorite 6. These observations indicated that it might be worth while to test the responses of celery seed to such compounds.

The NaOCl treatment consisted of soaking the seed with several changes of water during a period of 16 hours, then treating for two hours in various strengths of NaOCl, the proportion of solution to seed being 20 : 1 by weight. After brief rinsing and drying, four replications of 100 seeds were sown in

washed sand in the greenhouse. The results are presented in figure 2. While there was no significant difference in the total germination, the lots treated with NaOCl germinated and emerged significantly sooner than the untreated controls or those having water only.

Variety Tall Utah responded well to NaOCl solutions of ½% to 2% "available chlorine", and 1% was adopted as standard for this variety. On variety Detroit Golden, ½% NaOCl gave best results and the total germination was reduced somewhat by the 1½% and 2% solutions tolerated by Tall Utah. Varying the period of washing prior to hypochlorite treatment showed little if any difference for periods of four to 24 hours.

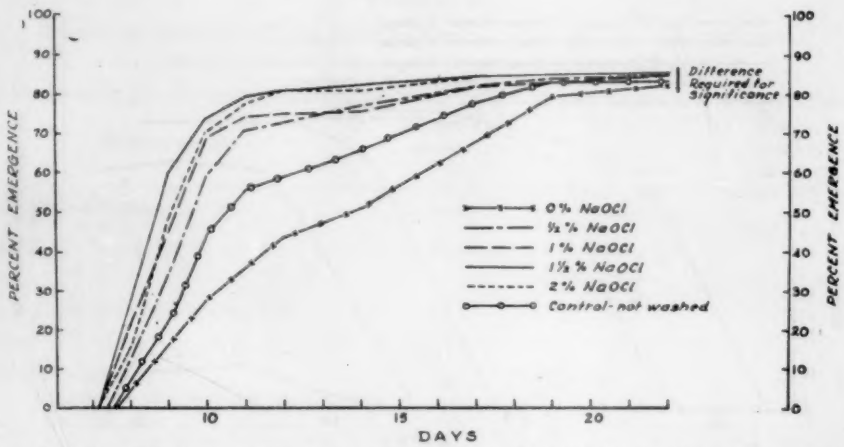

FIG. 2. Rates of emergence of untreated and hypochlorite treated celery seed in greenhouse sowings; daily range of temperatures, minimum 70° F. and maxima 80–96° F.

While the hypochlorite treated seed germinated rapidly in the greenhouse, it germinated slowly in outdoor sowings where lower night temperatures prevailed. The data are omitted since they showed essentially the same difference in speed of germination as that indicated by a comparison of figures 2 and 3.

Since it has been shown that the principal purpose of presprouting other seed **5, 4,** was to keep it under favorable germination conditions for as long as possible, the presprouting of celery seed was suggested.

Holding moist seed *en masse* for initiating germination prior to sowing obviously should be done in such way that all of the seeds approach the sprouting time as uniformly as possible. Therefore a series of trials were made to determine which of the environmental factors need be rigidly controlled, and the effects of their deviation on rate of germination. Since the sodium hypochlorite treatment had been found beneficial to germination as well as useful for controlling molds in the confined moist seed, most of the work was done with this class of seed, untreated seed being used occasionally for comparison.

Figure 3 shows the rates of emergence obtained from outdoor sowing of untreated and hypochlorite treated seed, following various periods of presprouting in light at room temperature. It includes a sample which was presprouted and re-dried prior to sowing. The daily range of temperatures was 36–60° F. for the first two weeks and somewhat warmer thereafter. The hypochlorite treated seed germinated to higher percentage than the untreated seed; the hypochlorite treatment alone speeded up emergence by several days; and hypochlorite treatment plus 5 days presprouting caused emergence nine to ten days earlier than the untreated seed. In fact, emergence from the latter class of seed was nearly complete before any

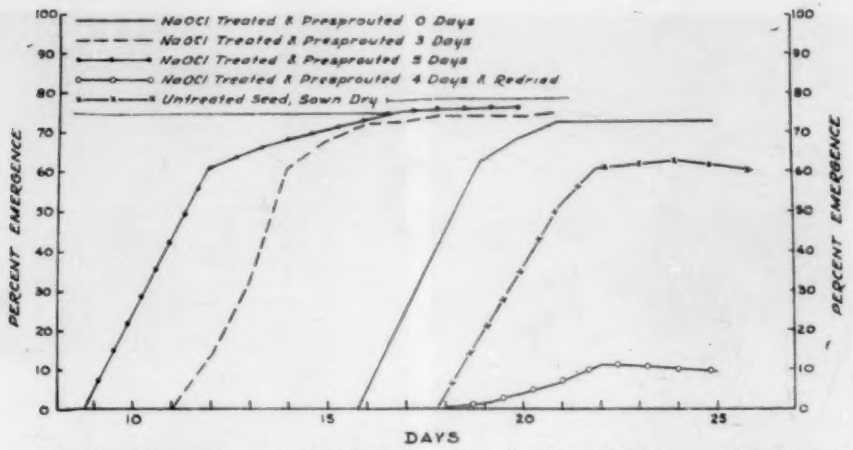
NaOCl Treated & Presprouted 0 Days
NaOCl Treated & Presprouted 3 Days
NaOCl Treated & Presprouted 5 Days
NaOCl Treated & Presprouted 4 Days & Redried
Untreated Seed, Sown Dry
PERCENT EMERGENCE
DAYS

FIG. 3. Rates of emergence in outdoor sowings of hypochlorite treated celery seed presprouted for various periods. Prevailing daily range of temperatures 36–60° F. during the first two weeks and somewhat warmer thereafter.

emergence had occurred from the untreated seed. Twenty-four hours drying of seed presprouted for four days reduced emergence to 11%.

Celery seed is responsive to light in its germination, therefore if large quantities are presprouted in the light those at the surface will begin their germination ahead of those in the center of the mass. Since the purpose of presprouting is to insure a rapid and uniform rate of germination in the field, presprouting in the light might tend to cause the opposite effect. Therefore it seemed necessary to presprout the seed in the dark but under conditions which would give a rapid and uniform rate of germination.

MORINAGA **2** has shown that in the absence of light the total germination of celery seed is greater at a constant temperature of 50° F. than at the other constant temperatures tried; and is almost completely inhibited at constant temperatures of 70° and 80° F. He also showed that by using alternating temperatures, the maximum germination could be obtained; but no results were reported on the effect of temperatures on the rate of germination nor the effect of varying the time of exposure to the higher

temperature. To obtain more information on the rate of germination as affected by different temperatures, celery seed was germinated in the dark at temperatures indicated in table I.

Thus it appears that two hours daily at 50° F. was sufficient to relieve most of the inhibiting effects of continuously warm temperatures; and two hours daily at 70° F. was sufficient to effect vigorous complete germination of seed held at 50° F. the remaining 22 hours daily. Highest percentages of germination were attained when the low temperature was maintained for at least eight hours daily.

TABLE I

EFFECT OF TEMPERATURE FOR VARYING PERIODS DAILY ON THE RATE AND TOTAL GERMINATION OF HYPOCHLORITE TREATED CELERY SEED.

TEMPERATURE	INITIAL SPROUTING @-DAYS	SPROUTING COMPLETED @-DAYS	TOTAL GERMINATION PERCENT.
80° F. constantly	none	none	0
70° F. "			4%
70° F. 22 hrs & 50° F. 2 hrs	6 days	14 days	60
20 " 4 "	6	14	70
16 " 8 "	7	16	78
8 " 16 "	8	16	87
4 " 20 "	9½	18	91
2 " 22 "	10	18	91
50° F. constantly	15½	26	87
Difference required for significance			6

Lengthening the daily period at low temperature lengthened the presprouting period, but did not materially lengthen the ensuing emergence period.

The above results apply only to germination in darkness. In even diffused light, the seeds germinated much more rapidly and at continuously high temperatures.

In another test, untreated and hypochlorite treated seed were germinated in weak light at a temperature of 66–72° F. and in darkness at alternating temperatures of 48° and 70° F. The results (fig. 4) show that treating the seed increased the rate of germination and that this alternating temperature could not entirely replace the effect of light in its effect on the rate of germination or total germination of the seed.

This test also included the germination of the hypochlorite treated seed in the dark at 48° F. and at alternating temperatures of 68° and 86° F. and at 48° and 70° F. (fig. 5). In darkness at 48° F. total germination was fairly high but the rate was very slow; while at 68–86° F. in the dark only five per cent. of the seeds germinated. In another test, several lots of seed which showed zero germination when held for 17 days in the dark at 70° F. germinated promptly when transferred to light.

Other environmental factors of presprouting which were investigated

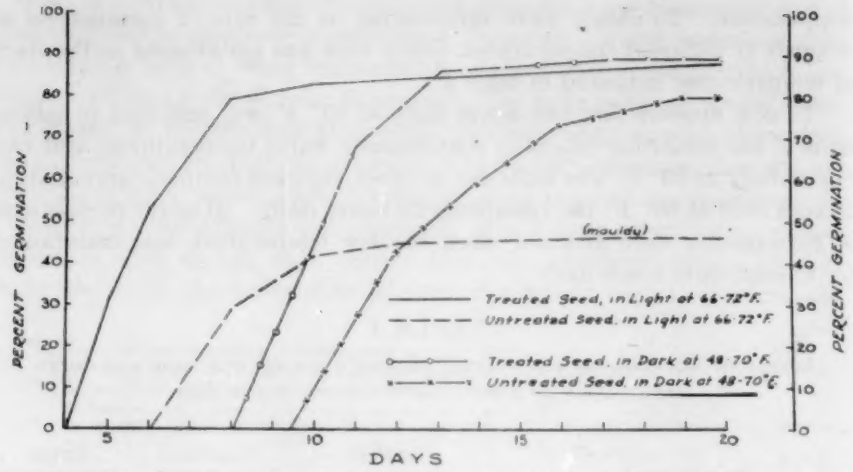

FIG. 4. Rates of germination of hypochlorite treated and untreated celery seed in light and dark at several alternating temperatures.

were: aeration as influenced by depth of the mass, daily inversion of the seed as from stirring, and the wetness of the seed. For this series of tests, hypochlorite treated seed was soaked, centrifuged to known water contents expressed as per cent. of the dry seed weight and held in darkness at alternating 48° and 70° F. temperatures.

For effects of depth of seed, test tubes were filled to ½", 2" and 5" with seed of 100% water content, and with seeds on moist filter paper for "Zero depth" controls. Loose fitting covers were used. At the end of nine days when first sprouting appeared, duplicate 100-seed samples from the material well mixed were transferred from each test tube to Petri germination at the same original temperatures and in the dark. The influence of depth of seed mass during presprouting on speed of germination is shown in figure 6.

While there was no significant difference in total germinations, the in-

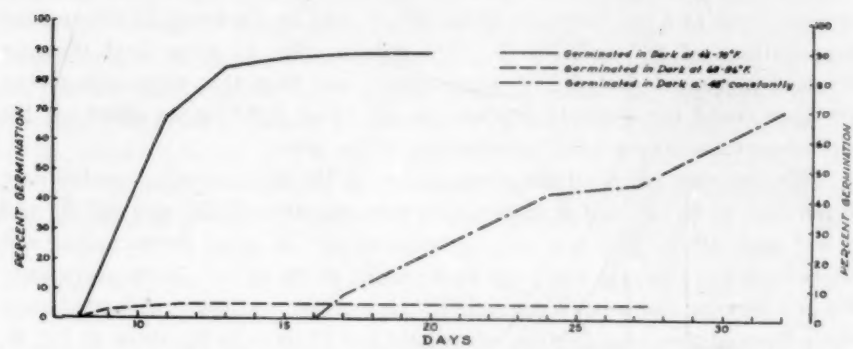

FIG. 5. Rates of germination of hypochlorite treated celery seed in the dark at high and low alternating and low constant temperatures.

creasing depths of seed mass during presprouting were associated with delay in starting germination and further lengthening of the germination period. The lot held five inches deep during presprouting required six days longer than the controls to attain 80% germination.

Daily movement of the seed two inches deep by stirring or inverting the containers during the presprouting period made no difference in the rate or total germination. Thus in instances where it is desirable to stir the seed to maintain uniform moisture or to facilitate aeration, the stirring should not be expected to retard presprouting or subsequent germination.

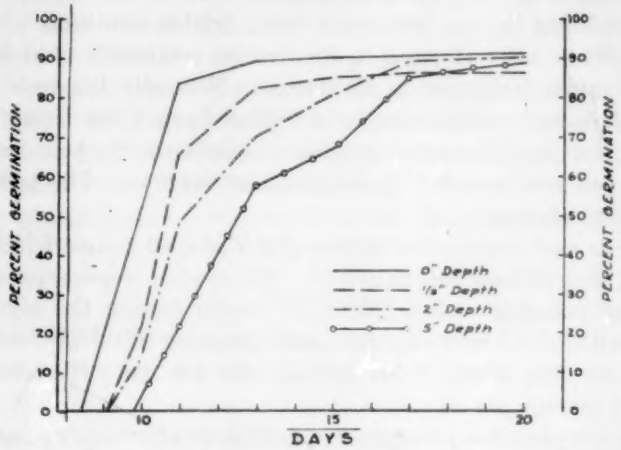

FIG. 6. Rates of germination of hypochlorite treated celery seed as affected by the depth of seed mass during prespouting.

When seed was presprouted at two inch depth without stirring and with water contents of 100%, 140% and 180% respectively, the only difference in germination observed was in the lot having 180% water. Seed from the upper portion of this lot germinated as well as the other two lots, but the bottom fourth of the lot which was soppy wet did not germinate during the ensuing 20 days. From a practical standpoint, the seed with 180% water was also much too wet to sow in a drill. That having 140% water could possibly be drilled, and that having 100% water was free flowing. When seed containing moisture somewhat in excess of 100% of the dry weight of the seed is held one to two inches deep in a chamber kept at high humidity (95–98%), little drying occurs so there is no necessity of adding water during the presprouting period.

FIELD TRIALS—With the foregoing experiments as a guide to methods of preparing the seed for direct seeding to the field, outdoor sowings were made to check on agronomic phases such as timing and frequency of irrigation, puddling of the surface soil as by rain, and the rate of emergence of hypochlorite treated presprouted celery seed. Its use exclusively for this trial seemed justified by the results of the previous tests reported herein.

The seed, celery variety Tall Utah 10-b, had been pre-treated by soaking 16 hours with several changes of water, held in 1% solution of sodium hypochlorite for two hours, rinsed, dried in light at room temperature and stored several weeks. Then it was soaked four hours in tap water and centrifuged to water content of about 100% of the dry seed weight. The moist seed was transferred to a dark chamber of high humidity and controlled temperature of 48° F. 16 hours and 70° F. for eight hours daily. When sown at the end of 8½ days, about 10 per cent. of the seeds were starting to sprout.

The ground used for the plots was very fine sandy loam, lightly pre-irrigated to bring the top foot into a damp friable condition. Flat topped ridges or "beds" were formed in the fashion commonly used for growing vegetables under irrigation in California. Normally the beds containing two seeding rows are separated by a shallow furrow for irrigation, but in this trial involving differential irrigation treatments, the beds were made so that each bed was bounded by independent furrows. The beds were not packed after shaping.

The seeds were sown in the bottom of a V shaped groove ¾ inch deep and ¼ inch to ⅜ inch of loose soil pulled in. The sowing was comparable to that obtained by planting with a Planet Jr. seeder having the covering shoes removed and with a length of small chain dragging in the groove to crumble in a light covering of soil. The seeding rate was 90 to 100 seeds per foot, or about 1½ pounds per acre.

Half of the plots were irrigated immediately after sowing and the other half 12 hours later. To check on the effects of puddling, as might occur when rain followed shortly after sowing, half of the plots had water applied to the surface of the seeding row at the rate of five ounces per foot of row.

Beds of each of the previous treatments were irrigated as follows: Once, following sowing; on the first and fifth days; the first, third and fifth days; and daily.

The first seedlings started to emerge at 3½ days, and at the end of 4½ days after sowing, there was an average stand of ten seedlings per foot. At 6½ days there were 39 seedlings per foot and at ten days the average stand was 46 per foot. Thus, those emerging the first four days were more than enough for a field culture stand, and most of the final stand was obtained during the first week. The prevailing daily temperatures were 55–68° F.

Stands were rather uniformly distributed within treatments except that wherever the edge of the bed occupied by the seeding row was low enough to permit flooding during irrigation, seedlings were almost nil. In those sowings deliberately puddled at time of sowing, stands were reduced by half.

Delaying the first irrigation until 12 hours after sowing did not delay germination nor reduce the number of seedlings. Also there was no significant difference in the speed of emergence or number of plants associated

with the frequency of irrigation. The plots which had only one irrigation did about as well as those irrigated more frequently. However the conditions in the experiment were more favorable than are likely to occur in the average large field sowing.

The seeding rate proved to be at least three times as heavy as was necessary for good field stand under these conditions. The density of stands obtained caused the plants to be leggy until they were thinned.

The quick emergence obtained by the above methods resulted in emergence of the celery plants as early as the most rapid growing weeds.

The above plot trials were supplemented by field sowings of from 50 to 100 acres near Salinas California for three years. The methods indicated by the experimental work were adopted by a grower who has obtained good stands at relatively low costs, and the methods have been found agriculturally and economically sound. In some instances, young plants resulting from thinning operations were transplanted to augment acreage with a later maturing crop. Celery seedlings are rather tolerant of oil sprays while still very young, so weed control was comparatively easy and economical.

Summary

Effects of light, alternating temperatures, hypochlorite treatment and other factors on the rate and total germination of celery (Apium graveolens) seed were studied.

The results suggested a method of presprouting the seed under controlled conditions prior to sowing outdoors. The method was found to reduce the preemergence period and greatly facilitate growing the species by direct seeding.

U. S. Rubber Research Field Station
Bureau of Plant Industry
Salinas, California

LITERATURE CITED

1. Rules and Regulations under the Federal Seed Act, Agricultural Marketing Administration, U. S. Dept. Agric., p. 13. 1940.
2. Morinaga, Toshitaro. Effect of alternating temperatures upon the germination of seeds. Amer. Jour. Bot. **13**: 141. 1926.
3. ————————. Germination of seeds under water. Amer. Jour. Bot. **13**: 126.
4. Zasiadnikov, T. Spring sowing of Kog-saghyz, Sotsialisticheskoe Sel'skoe Khozaistvo. **4**: 70–75. 1941. Trans. Dr. G. Krotkov, mimeo.
5. Taylor, Carl A. The Propagation of Guayule, Emergency Rubber Project, U. S. Dept. Agric., pp. 24–26, 30. 1946.
6. ————————. Ibid., pp. 15–16.
7. Benedict, Harris M., and Robinson, Jeanette. Studies on the Germi-

nation of Guayule Seed. Tech. Bull. 921, U. S. Dept. Agric. Oct., 1946.

8. DONEEN, L. D., and MACGILLIVRAY, J. H. Germination (emergence) of vegetable seed as affected by different soil moisture conditions. Plant Physiol. **18**: 524–529. 1943.
9. Seed and Nursery Operations Handbook, rev. May, 1946. U. S. Department of Agriculture, Emergency Rubber Project, mimeo, 1946.

LOCALIZATION OF PHOSPHORYLASE AND OF STARCH FORMATION IN SEEDS

H. C. YIN AND C. N. SUN

(WITH THREE FIGURES)

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Introduction

The discovery of the enzyme phosphorylase provides an important clue toward the solution of the problem of starch transformation in plants (**5**). The enzyme has been purified and its properties and reactions *in vitro* are fairly well understood (**7**) (**11**). Only its physiological role in the living plant remains obscure. One approach to this problem is to investigate the distribution of the enzyme in different tissues and cells to correlate it with the site of normal starch synthesis. For this purpose a histochemical method has been devised and described (**13**). With this method a number of observations have been made on various plant parts. The present report embodies the results of the work on germinating seeds.

Briefly the method for the detection of phosphorylase (**13**) consists of incubating free-hand sections of plant parts in a buffered medium of glucose-l-phosphate (prepared according to the method of HANES (**6**) and of subsequent staining with iodine in potassium iodide. The amount of starch formed is taken as a measure of the phosphorylase activity. Incubation is conducted at 25° C and lasts for a half to one hour, or longer in case the enzymic activity is particularly weak.

Soybean was chosen as the main experimental material because of the absence of detectable starch, which interferes with the test, in the dormant seeds and during the first days of germination. Castor bean which is also practically starch-free was also tried. For comparison, broad bean was used, but in this case the embryo was removed from the cotyledons and starved for several days to rid it of stored starch.

Distribution of Phosphorylase

The results on the distribution of phosphorylase activity are presented diagrammatically in figure 1.

In the soybean (fig. 1, A and B) the most intense reaction occurs in the root cap, less in the root tip and the lateral buds and still less in the hypocotyl and the stem tip. Very little activity is found in the cotyledons and is confined to the surface cells of the middle portion. There are wide differences of reaction intensity in the individual cells. Some cells are practically filled with starch so that the whole cell stains dark with iodine, some cells are partially filled, and still others contain only a few isolated starch grains (fig. 3, A). These differences are represented in the diagrams (fig. 1) by

the size of the dots. It is seen that cells of the first type are mostly found in the root tissues and only that of the last type are present in the cotyledons.

Similar distribution of phosphorylase activity is shown by the embryo

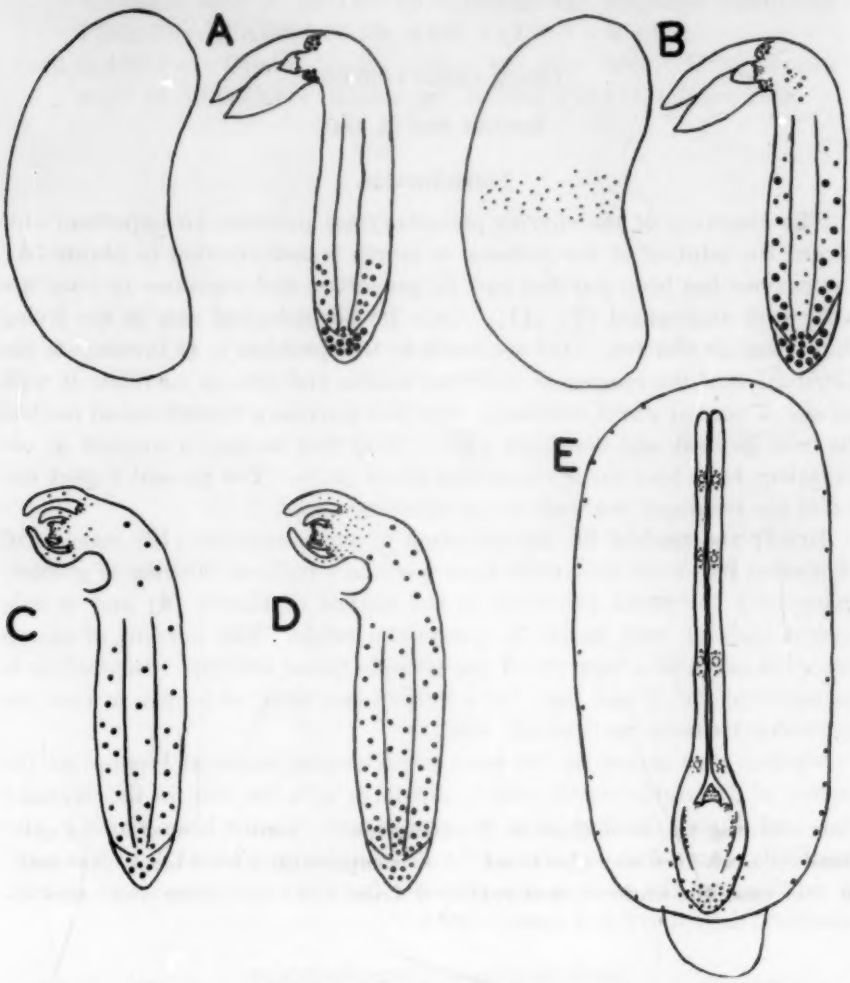

FIG. 1. Distribution of phosphorylase activity in seeds. A and B. cotyledon and embryo of soybean one and two hours after incubation respectively. C and D. embryo of broad bean one and two hours after incubation. E. tissues of castor bean, 12 hours after incubation. Size of dots indicates relative intensity of reaction of the cells, as explained in the text.

of the broad bean (fig. 1, C and D). More intense reaction is located in the root and the hypocotyl than in the young leaves and stem meristems. Unlike the soybean, the individual cells are more uniform in their activity. No experiments have been made with the cotyledons, because they contain much stored starch which renders the test inapplicable.

Very weak activity is found in the tissues of the castor bean. Starch is detectable only after prolonged incubation (3–12 hours). It is mainly confined to the root and stem meristems and near the vascular bundles in the cotyledons. Faint reaction is also shown by isolated cells in the peripheral portions of the endosperm (fig. 1, E).

Localization of Starch Normally Present in the Seeds

In order to ascertain the part played by phosphorylase in the normal physiological process of starch formation in the seeds, the distribution of the enzyme activity is compared with that of starch accumulation in the seeds before and after germination (**10**). Seeds were soaked in water for 12 hours and allowed to germinate at 25° C. Free-hand sections were made at daily intervals, fixed and stained with iodine in potassium iodide. The results are given in figure 2.

In general no starch can be detected in the dormant soybean; it is formed only after germination (fig. 2, A and B). The synthesis of starch appears to be most active in the root cap, less in the root tissues, the hypocotyl and the cotyledons, still less in the stem meristems and very slow in the young leaves. The phenomenon is especially clear in seeds germinated at lower temperatures. The appearance of starch in the cotyledons is earlier by one to two days than in the stem tip. Quantitatively the starch formed in normal development is always less than that formed in sections incubated in glucose-l-phosphate. In no case, are cells found to be filled with starch (fig. 3, B). The starch grains are compound; each leucoplast contains several loci.

A few observations were also made on the broad bean. Both the embryo and the cotyledons contain starch even before germination. Figure 2, D shows that most of the starch is present in the cotyledons and the hypocotyl, less in the stem and root tissues and none in the young leaves. The starch grains are in general very large and few in number. Frequently a single starch grain almost fills up a cell (fig. 3, F).

In the castor bean, starch is found only after germination is far advanced. It is concentrated in the root and the tip portions of the endosperm (fig. 2,E).

From the above results it may be concluded that the site of normal starch formation coincides, in general, with that of phosphorylase activity, indicating that the enzyme plays an important role in the starch synthesis during germination. There are, however, minor discrepancies. For example, in the soybean starch is formed earlier in the cotyledons than in the stem tissues although the latter is more active in phosphorylase reaction, and in the broad bean less starch is found in the root cap and root tip and none in the young leaves as the activity of phosphorylase would demand (compare figs. 1 and 2). Furthermore, in the individual cells, less starch is always present in normal developing seeds than that in sections supplied with glucose-l-phosphate (fig. 3, A and B) and a smaller number of starch grains are visible (fig. 3, E and F). These differences suggest that there are factors other than phosphorylase involved in normal starch formation. The avail-

ability of substrate and the efficiency of the phosphorylating mechanism, for instance, may at times, be limiting (2) (7).

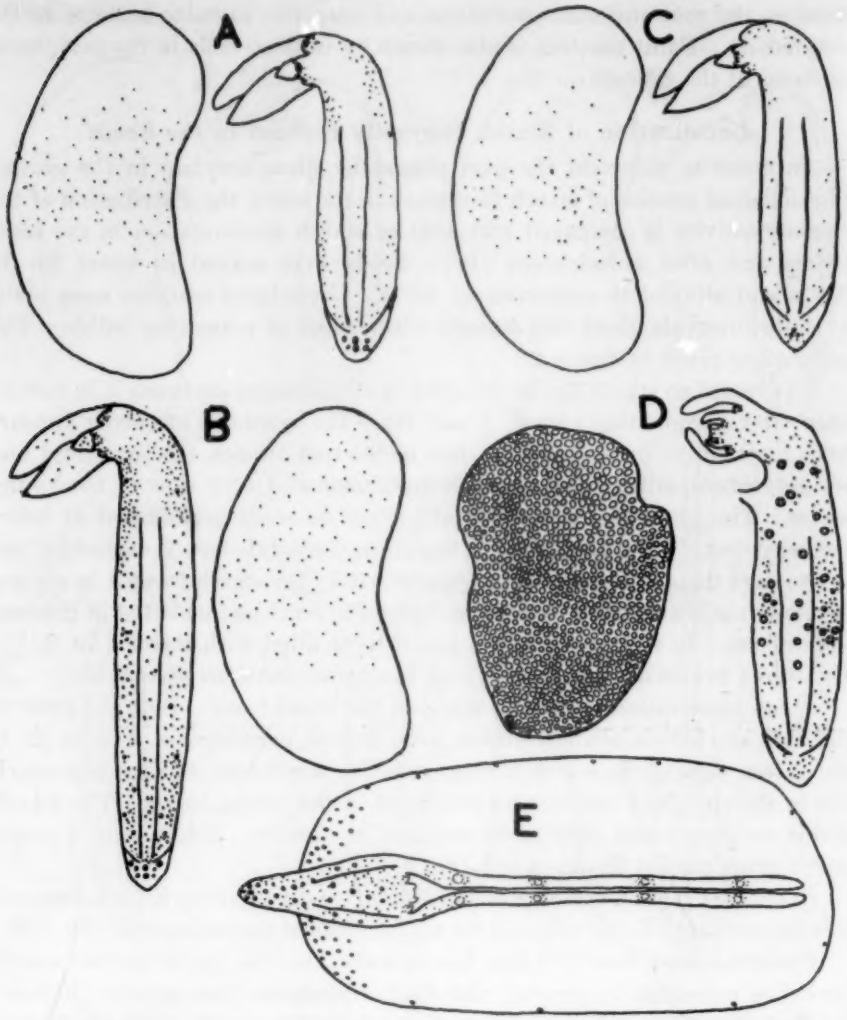

Fig. 2. Starch normally present in seeds. A and B. Cotyledon and embryo of soybean, one and two days after germination. C. Same, after soaking and boiling, or after incubating 24 hours in glucose. D. Cotyledon and embryo of broad bean after soaking for 12 hours in water. E. Castor bean, seven days after germination. Size of dots indicates relative amount of starch present, as in figure 1: circles represent groups of cells with single big starch grains as shown in figure 3, F.

Experiments with Glucose and other Sugars

In order to obtain an insight into the phosphorylating mechanism of the seeds, several experiments were made with sections of soybean incubated in glucose or other sugars (sucrose, galactose, levulose, mannose, etc.) in

phosphate buffers with or without the addition of magnesium sulfate and adenosine-triphosphate. The results are exemplified by figure 2, C. It may be seen that only very little starch can be detected even after prolonged

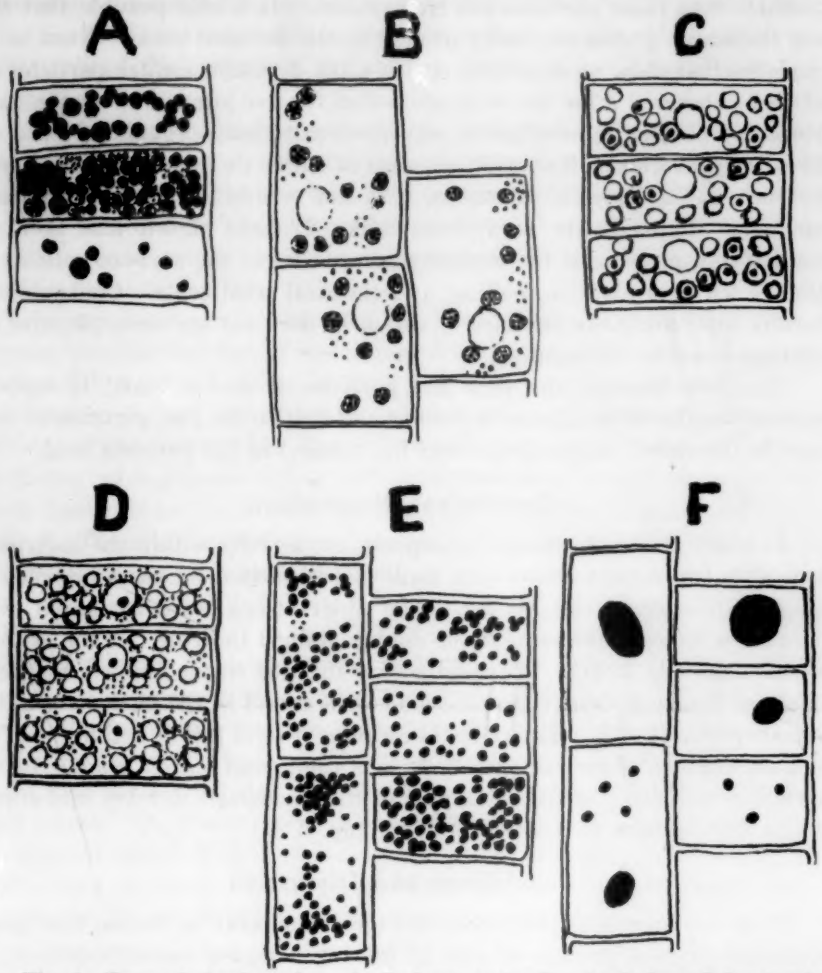

Fig. 3. Form of starch grains. A. Soybean, root cells, starch formed due to phosphorylase activity after incubation in glucose-1-phosphates. B. Same, starch formed after germination. C. Same, starch developed after soaking and boiling. D. Same, leucoplasts (circles) and mitochondria (small dots). E. Broad bean root cells, starch formed due to phosphorylase activity. F. Same, starch normally present before starving.

incubation (24 hours). The appearance of starch is comparatively uniform in both the cotyledon and the embryo. These results show that under the conditions of these experiments, the sections of tissue are unable to utilize or phosphorylate glucose with the formation of starch (**2**). Indeed, it is doubtful that even the little starch observed comes from the added sugar at all.

Some varieties of soybean after soaking and sectioning do show fine particles stainable with iodine (fig. 3, C). Due to their small size, their color cannot be clearly defined under the ordinary microscope. It is questionable that these particles are truly starch. It is also possible that they are the starch grains originally present in the dormant seeds. Even in the varieties that show no detectable starch when dormant, similar particles can often be observed after the seeds are boiled for five minutes or treated with bleaching powder, mineral acids, polyvalent salts, some organic solvents, etc. It is likely, therefore, that small amounts of starch do exist in dormant seeds but because of its colloidal status, it is not readily detected by ordinary methods. Experiments on soybean milk (**8**) have shown that both the emulsifying power and the isoelectric properties of the soybean colloids do change with germination, boiling, and chemical treatments. Contradictory results concerning the presence of starch in dormant soybeans (**9**) also encourage a similar explanation.

For these reasons, the very fine particles of starch found in soybean sections incubated in glucose or sucrose and that in the just germinated seed may be the starch originally present but masked in the dormant seed.

Cytological Observations

To study the localization of phosphorylase activity within the individual cells, thin free-hand sections were made and incubated for short intervals in glucose-l-phosphate (fig. 3, A). The observations were compared with paraffin sections of the same tissue doubly stained to bring out the cellular constituents (fig. 3, D). The results show that the enzyme is present exclusively in the leucoplasts. No reaction can be found in the nucleus or in the mitochondria (**1**) (**3**). Each plastid contains several loci of activity. After long incubation, these loci fuse to form a compound starch grain. In the normal germinating seeds only a part of the leucoplasts develop into starch grains due perhaps to lack of substrate (fig. 3, B).

Conclusions and Discussion

From the present investigation the conclusion may be drawn that phosphorylase plays a prominent role in the physiological starch synthesis in germinating seeds. Evidence comes from two sources. Firstly, the distribution of the enzymatic activity coincides in general with that of normal starch formation. Secondly, the reaction is exclusively confined to the plastids where starch is normally accumulated.

The localization of phosphorylase activity and of starch formation in the root and stem tips indicates that the enzyme is connected with active growth and that starch is a temporary food reserve of the active developing cells. It is readily formed when the raw material is available and quickly consumed during starvation. Even oily seeds contain phosphorylase and accumulate starch in their growing regions. The presence of the enzyme in the root cap shows that this organ is not simply a protective structure but is an active

center of metabolic changes. The concentration of other enzymes, e.g., phosphatase (**4**) in this region also supports this contention. In the castor bean, phosphorylase is found near the vascular tissues indicating that the enzyme has a probable function in translocation (**12**). Unfortunately the present method is not applicable to plant parts that contain stored starch so that the role of the enzyme in the storage organs cannot be similarly investigated.

Unlike the other enzymes so far studied cytologically (**1**), phosphorylase is shown to be exclusively confined to the plastids. In the germinating seed it is found in the leucoplasts, which may turn green in light. In the leaves (**14**) it is localized in the chloroplasts of both the guard cells and the mesophyll. These results supply a conclusive mechanism by which starch is formed in the plastids.

The minor descrepancies between the histological distribution of phosphorylase reaction and that of starch formation indicate that there are other factors which may be limiting in some tissues. It may be the availability of substrate or the activity of the phosphorylating mechanisms (**2**) or other conditions of the tissues (**7**). The failure to obtain starch formation in sections incubated in glucose with or without adenosine-triphosphate may mean that the former is not the normal raw material or the latter does not permeate the tissue. The evidence is, however, not conclusive and optimal experimental conditions may not have been obtained.

Summary

The distribution of phosphorylase activity and of starch formation in the soybean and other seeds was studied histochemically by the staining techniques. Phosphorylase reaction is most intense in the root cap, less in the root tip and the lateral buds, still less in the hypocotyl and the stem tip and very little in the cotyledons. Upon germination, starch is found first in the young leaves. The general coincidence of the sites of phosphorylase activity with those of starch formation indicates that the enzyme is responsible for the synthesis of starch during germination. Minor discrepancies are perhaps due to the availability of the substrate, the efficiency of the phosphorylating mechanisms or other conditions in the tissues. Cytological observations show that phosphorylase is exclusively localized in the plastids. These observations further confirm the contention that phosphorylase is the normal physiological mechanism of starch formation.

BOTANY DEPARTMENT,
NATIONAL PEKING UNIVERSITY,
PEKING, CHINA.

LITERATURE CITED

1. BOURNE, G. Cytology and cell physiology. Oxford, 1941.
2. COLOWICK, S. P., and SUTHERLAND, E. W. Polysaccharide synthesis from glucose by means of purified enzymes. Jour. Biol. Chem. **144**: 423–437. 1942.

3. Danielli, J. F., and Catcheside, D. G. Phosphatase on chromosomes. Nature **156**: 294. 1945.
4. Glick, D., and Fisher, E. E. Studies in histochemistry. XVII. Localization of phosphatases in the wheat grain and in the epicotyl and roots of the germinated grain. Arch. Biochem. **11**: 65–87. 1946.
5. Hanes, C. S. The breakdown and synthesis of starch by an enzyme system from pea seeds. Proc. Roy. Soc. B. **128**: 421–450. 1940.
6. ————. The reversible formation of starch from glucose-1-phosphate catalyzed by potato phosphorylase. Proc. Roy. Soc. B. **129**: 174–208. 1940.
7. Kalckar, H. M. The chemistry and metabolism of the compounds of phosphorus. Ann. Rev. Biochem. **14**: 283–308. 1945.
8. Lou, C. H., and Chen, S. L. Unpublished results.
9. Markley, K. S., and Goss, W. H. Soybean chemistry and technology. New York, p. 25. 1944.
10. Von Ohlen, F. W. A microchemical study of soybean during germination. Amer. Journ. Bot. **18**: 30–49. 1931.
11. Wyme, A. M. Non-oxidative enzymes. Ann. Rev. Biochem. **15**: 35–74. 1946.
12. Yin, H. C. A histochemical study of the distribution of phosphatase in plant tissues. New Phytologist **44**: 191–195. 1945.
13. ————, and Sun, C. N. Histochemical method for the detection of phosphorylase in plant tissues. Science **105**: 650. 1947.
14. ————, and Tung, Y. T. In press.

CARBON AND NITROGEN BALANCE OF CHLORELLA DURING GROWTH

JACK MYERS AND JAMES A. JOHNSTON

(WITH ONE FIGURE)

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In studies on the algae remarkably little attention has been paid to those details of metabolism which have proved essential in understanding the physiology of other microbial forms. On the carbon and nitrogen balance, for instance, there is little available information. KROGH, LANGE, and SMITH (**3**) obtained a carbon balance on a culture of Scenedesmus which showed that 95% of the carbon was retained by the cells under the usual conditions of algal culture. BARKER (**1**) made tests for but failed to find soluble photosynthate which might be excreted by diatoms. One report of excreted photosynthate is that of UHVITS (**11**) who found that very small amounts of acetaldehyde of the order of 10^{-6} to 10^{-5} moles could be obtained from 200 to 700 ml. of algal suspension (*C. pyrenoidosa*) when bisulfite had been added to the medium. It is also common observation that algal cultures develop small amounts of bacterial growth even in completely inorganic media. More complete information on the carbon and nitrogen balance is needed.

The metabolism of the algae has been studied almost exclusively by the Warburg technique, which has obvious advantages for such work. There are also certain limitations, in particular a practical restriction to short-time experiments. It has seemed desirable to devise methods for the study of algal metabolism during growth in cultures.

Carbon Balance During Growth in the Continuous-Culture Apparatus

METHOD

The algal continuous-culture apparatus (**8**) allows maintenance of a constant density of population by a photometric dilution device which adds fresh medium at a rate equivalent to the rate of growth. In addition to other applications already described (**4, 5, 6**) the apparatus may also be adapted to metabolic studies. If the density of population is held constant, then the net uptake of any component of the nutrient medium by the cells per unit volume must equal the difference in the concentration of that component between the original medium and the final supernatant. This principle has been applied to the study of glucose assimilation in the dark according to the following procedure.

The photometric control was operated by an intensity of about four

foot-candles, which is below the compensation point for growth. The chamber, constructed essentially as the jacket of a glass condenser, was darkened by an opaque black paper wrapping except for a small window to the photocell. Since less than 1% of the culture was exposed by the window, the total illumination received by the culture was negligible.

The culture medium was a Knops solution containing KNO_3, KH_2PO_4, $MgSO_4$, iron, microelements, and citrate as previously described (**4**) and with 0.5% glucose added. Citrate is assimilated only slowly by Chlorella. The citrate concentration was less than 1% of the glucose concentration and may be considered negligible. The chamber was sterilized and inoculated with a pure culture of *Chlorella pyrenoidosa* (Emerson's strain). The photometric device was adjusted to maintain a population of about 0.75 mg. dry weight of cells per ml. (equivalent to about 3.0 cmm. cells/ml.). At the temperature of 25.0° C these conditions allow a relative growth rate per day of 0.55, i.e., $\log N/N_0 = 0.55$ t. This is equivalent to a multiplication of 3.5 times per day. The pH of the original medium was 5.0, of the harvested suspension, 6.2.

At approximately 24-hour intervals the culture was harvested, leaving a standard inoculum for the following day. The harvest was weighed to obtain the total volume and duplicate 50.0 ml. aliquots centrifuged. The supernatant was saved for glucose analysis; the cells were washed twice in water and dried *in vacuo* to constant weight to allow estimation of the total dry weight of cells in the harvest. The cells from the several experiments were pooled for analysis. Elementary analysis of the dried cells (by Dr. Carl Tiedcke, New York) yielded 7.34% H, 49.4% C, 8.50% N; unfortunately the ash determination was lost. Aliquots of the harvested supernatant and of the original culture medium were subjected to glucose analysis by the Shaffer-Somogyi method (**10**).

The culture was aerated with CO_2-free air scrubbed through three tubes of KOH and one of $Ba(OH)_2$. The effluent air from the culture was bubbled through a standard aliquot of $Ba(OH)_2$ in a Milligan gas-washing bottle. Absorption of carbon dioxide was carried over the same time interval required for growth of the daily harvest. At the end of the interval the $Ba(OH)_2$ was back titrated in the bottle with 0.25 N HCl to a phenolphthalein endpoint in order to estimate the total carbon dioxide produced.

Results

The data obtained from the four complete experiments are summarized in table I in terms of milligrams of carbon. An average recovery of 93% of the used glucose carbon was obtained in cellular carbon and carbon dioxide. Errors in the method which might make the observed recovery too high could be due to the effects of the small illumination of the photometric device and to the possibility of some assimilation from citrate. It is also possible that some reducing substance may have been excreted and measured

as glucose although no such excretory products could be found in cultures growing photosynthetically. On the other hand, all such recovery experiments are subject to some losses. In the present work some unavoidable loss of carbon occurs as unmeasured carbon dioxide in the harvested suspension and as loss of respiratory carbon dioxide from the cells during the initial stages of drying. The expected carbon losses are judged to be at least equal to the unmeasured gains from illumination and citrate. The recovery of 93% may be taken to indicate a nearly complete conversion of glucose to carbon dioxide and cellular material.

TABLE I

CARBON BALANCE DURING GLUCOSE ASSIMILATION IN DARKNESS

MG. CELLS	MG. CELL CARBON	MG. CO_2 CARBON	MG. CELL + CO_2 CARBON RECOVERED	MG. GLUCOSE CARBON USED	% RECOVERY	% GLUCOSE CARBON ASSIMILATED
116	57.4	51.6	109.0	117	93	49
129	63.6	52.0	115.6	125	92	51
104	51.4	47.1	98.5	110	90	47
126	62.2	55.5	117.7	124	95	50
				Average	93	49

The assimilation of 49% of the glucose carbon to cellular carbon is much lower than the 83% calculated from manometric experiments on starved cells of Chlorella (**7**). It must be remembered, however, that the present data describe growing cells in which assimilation often proceeds less efficiently than in resting cells (cf. 12). It will also be shown elsewhere that nitrate reduction and assimilation, which do not occur in starved cells, must proceed rapidly during growth with increased carbon dioxide production and hence less complete assimilation of glucose (**2**). Investigation of the completeness of glucose assimilation during ammonia assimilation would be of particular interest but has not yet been attempted.

Comment may also be made on the usefulness and limitations of the above procedure. An inherent advantage is that the cells are maintained under constant physiological conditions. For example, even though the cells are consuming glucose, the glucose concentration in the medium is maintained constant. The method is not limited to glucose; it may be extended to any substrate, growth factor, or poison and applied to almost any unicellular microorganism. A limitation of the method, as used here for Chlorella, is that it was not adapted to the study of metabolism during photosynthesis. For constant flow of air it was necessary that the influent air contain zero carbon dioxide. An air recirculation method in conjuction with absorption gas analysis was considered too elaborate for present purposes. A second and entirely different method was therefore devised.

Carbon and Nitrogen Balance During Growth in Mass Cultures

Method

A nine-liter serum bottle containing one liter of Knops solution (as above but without glucose) was sterilized by autoclaving and inoculated with a known amount of cells of *C. pyrenoidosa* from the continuous-cul-

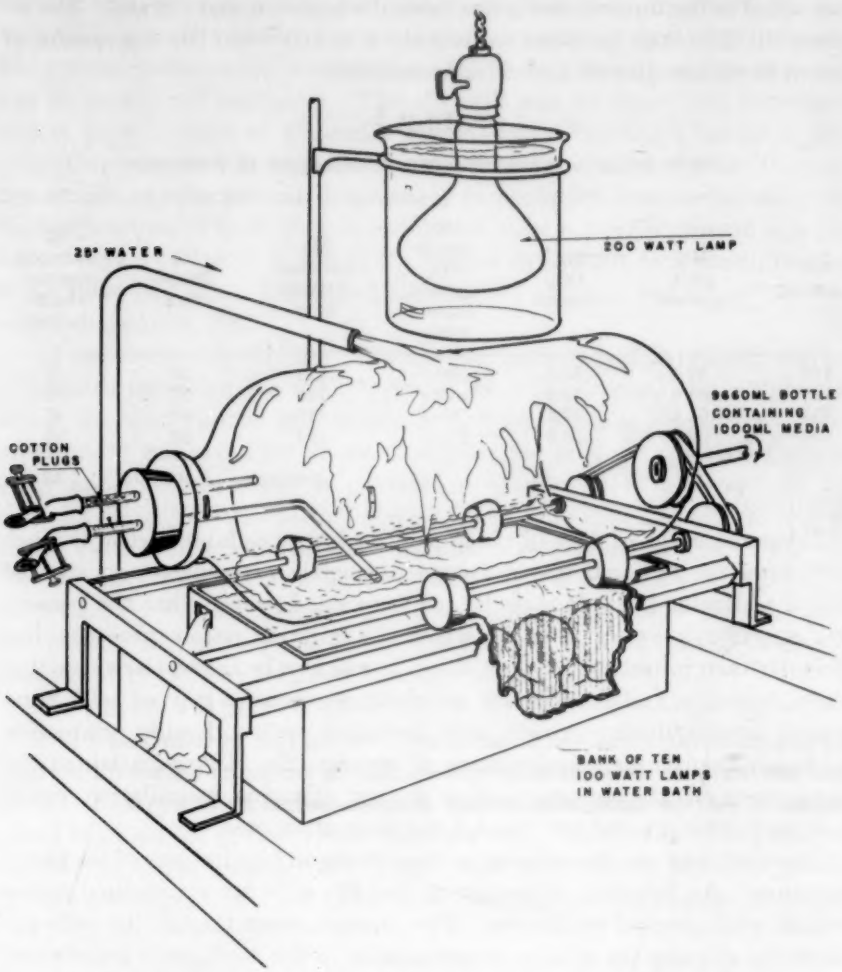

Fig. 1. Apparatus for metabolic studies on mass cultures of algae.

ture apparatus. The rubber stopper with inlet and outlet tubes was then wired down and waxed. A tank mixture of 5.5% carbon dioxide in air was bubbled through the darkened bottle overnight. Analyses for CO_2 and O_2 in the bottle air were made with a Haldane apparatus. The bottle was then placed in position and flooded with thermostated 25.0° C water. The pressure in the bottle was determined with a capillary manometer and the barometric pressure noted.

The lights were then turned on and the bottle rotated on rollers to give the common roll culture. A high light intensity of about 500 fc was provided by a bank of ten 100-watt lamps set in a water bath which extended about 3 cm. above the bulbs and provided an infra-red filter. For a low light intensity of 50 fc, two 60-watt bulbs were used underneath the bath. The experimental set-up is shown in figure 1.

At the end of the experiment (two–four days) the bottle pressure and barometric pressure were again determined and CO_2 and O_2 analyses made on the contained gas. The volume and pH of the suspension were determined. The cells from several aliquots of suspension were centrifuged out, washed in water, dried *in vacuo*, and the total dry weight thus estimated. The centrifuged supernatant could be used for analysis of any of the components of the medium and the dried cells could be subjected to analysis.

The gas exchange was calculated from the initial and final gas analyses and corrected for amounts of CO_2 and O_2 dissolved in the liquid. From the pH of the suspension an additional correction had to be made for the CO_2 bound as bicarbonate. The details of calculation for a typical experiment are indicated in table II.

Nitrogen uptake was measured as the difference in nitrate concentration between the original medium and the harvested supernatant. For convenience in analysis the nitrate concentration in the Knops solution was reduced to one-half the usual value so that each culture initially contained 6.44 millimols of nitrogen. In the course of an experiment about half of the nitrate was assimilated. The determination was made on a 25 ml. aliquot by alkaline reduction with Devarda alloy, distillation into 8 ml. of 0.025 N HCl, and back titration of the acid with 0.02 N KOH using a calibrated microburette and a glass electrode.

Results

Our first application has been a study of carbon and nitrogen balance of Chlorella during photosynthetic growth. The carbon used was estimated from the CO_2 taken up by the cells in the course of the experiment. The carbon recovered in the cells was estimated from the total dry weight of cell harvest (minus the small amount of inoculum) times the carbon content found in the cells by elementary analysis. The pooled cells of three experiments at low light intensity and three experiments at high light intensities yielded analyses:

Low Light Intensity: 7.32% H, 49.80% C, 8.56% N, ash analysis lost.

High Light Intensity: 6.93% H, 45.04% C, 8.70% N, 8.2% ash.

The analysis on low-light cells is remarkably similar to that obtained for cells grown on glucose in the dark; unfortunately, the ash determination again was lost. The ash analysis on the high-light cells was unusually high (Chlorella usually runs about 5% ash) and leads to some uncertainty in the whole analysis.

The carbon balance, summarized in table III, indicates a recovery of

TABLE II

SAMPLE CALCULATION (FOR EXPT. 6 OF TABLE III)

Temp. = 25.0° C. Vol. Suspension = 976 ml.

Initial Analysis: CO_2: 5.39% 5.39% O_2: 16.54% 16.53% pH 4.5

Barometric press. 747 mm.

Bottle press. − 2

V. press. water 24

Dry gas press. 721 mm.

$$8684 \times \frac{721}{760} \times \frac{273}{298} = 7550 \text{ ml. dry gas STP}$$

Gas phase: O_2 $0.1654 \times 7750 = 1250$ ml.
CO_2 $0.0539 \times 7750 = 407$ ml.

Liquid phase:

$$O_2 \frac{0.1654 \times 721}{760} \times 0.0283 \times 976 = 4.3 \text{ ml.}$$

$$CO_2 \frac{0.0539 \times 721}{760} \times 0.759 \times 976 = 37.9 \text{ ml.}$$

Bound CO_2 $\log \frac{[HCO^-_3]}{[CO_2]} = 4.5 - 6.33$

CO_2 (as HCO^-_3) = 0.06 ml.

TOTAL O_2 = 1254 ml.

TOTAL CO_2 = 445 ml.

Vol. Gas = 9660 − 976 = 8684 ml. $a_{O_2} = 0.0283$ $a_{CO_2} = 0.759$

Final Analysis: CO_2: 1.42% 1.43% O_2: 22.02% 22.05% pH 6.2

Barometric press. 752 mm.

Bottle press. − 4

V. press. water 24

724 mm.

$$8684 \times \frac{724}{760} \times \frac{273}{298} = 7580 \text{ ml. dry gas STP}$$

Gas phase: O_2 $0.2204 \times 7580 = 1670$ ml.
CO_2 $0.0142 \times 7580 = 108$ ml.

Liquid phase:

$$O_2 \frac{0.2204 \times 724}{760} \times 0.0283 \times 976 = 5.8 \text{ ml.}$$

$$CO_2 \frac{0.0143 \times 721}{760} \times 0.579 \times 976 = 10.0 \text{ ml.}$$

Bound CO_2 $\log \frac{[HCO^-_3]}{[CO_2]} = 6.2 - 6.33$

CO_2 (as HCO^-_3) = 7.41 ml.

TOTAL CO_2 = 125 ml.

TOTAL O_2 = 1676 ml.

about 95% of the CO_2-carbon as cellular carbon at both low and high light intensities.

The nitrogen balance again indicates a recovery in the cells of about 95%. It was also shown that excretion of ammonia by the cells was not appreciable. Alkaline distillation of the supernatant without reduction never yielded more than about 0.1% of the total nitrogen determined with reduction. Micro-colorimetric determination by Nessler's reagent also showed only negligible traces of ammonia production.

TABLE III

CARBON AND NITROGEN BALANCE AND CO_2/O_2 QUOTIENT DURING PHOTOSYNTHETIC GROWTH

Expt.	Nitrogen			Carbon			CO_2/O_2 exchange STP		
	Mg. Used	Mg.* Rec'd.	% Recovery	Mg. Used	Mg.* Rec'd.	% Recovery	Ml.CO_2 Used	Ml.O_2 Produced	$\frac{CO_2}{O_2}$
				Low Light Intensity (50 fc)					
1	32.2	31.5	98	195	183	94	363	522	0.70
2	39.6	38.3	97	229	223	97	426	608	0.70
3	37.5	37.6	100	233	219	94	432	604	0.72
				High Light Intensity (500 fc)					
4	45.0	42.6	95	218	220	101	405	544	0.75
5	36.0	31.6	88	181	163	90	336	442	0.76
6	32.5	32.9	101	172	170	99	320	422	0.76

* Corrected for inoculum which contained 1.4 mg. C and 0.25 mg. N.

The experiments have also allowed measurement of the CO_2/O_2 exchange quotient during photosynthetic growth (table III). The data at the low light intensity of 50 fc, which is light-limiting for both growth and photosynthesis, confirm the quotient of 0.68 measured in short-time experiments by the Warburg method (**2**). The low value of the quotient has been shown to be the result of nitrate reduction. At high light intensity the short-time experiments by the Warburg method have yielded quotients of about 0.9 in the hands of many workers. The quotients obtained here during growth at light-saturating intensities are considerably lower, averaging 0.76. It now appears likely that the quotient of 0.9, so often reported for Chlorella, is not a stable value but describes a metabolism gradually adapting itself to high light intensity.

Discussion

It has been established that *Chlorella pyrenoidosa* is remarkably efficient in conserving photosynthetic products within the cells both at light-limiting and light-saturating intensities for growth. The overall product of photosynthetic metabolism is in new cells; excretory products are not appreciable.

The ecological significance of high cellular retention of assimilates by the algae has already been pointed out by KROGH, LANGE, and SMITH (**3**). In the biological system the green plant is not only the point of free energy

increase for carbon; it is also a point of decrease in entropy by virtue of its accumulation of carbon to a concentration many times greater than that of its environment. The role of the algae as biological accumulators would be much less important if they were to lose large amounts of photosynthate as organic excretory products.

We are still in search of an explanation of a phenomenon reported earlier (**5**). A maximum growth rate for Chlorella is attained at a much lower light intensity than is the maximum rate of oxygen evolution as measured in short-time manometric experiments. Two alternative explanations may be suggested: (1) that above light-saturation of growth there may occur an increased excretion of assimilates, or (2) that when first exposed to high light intensity the cellular metabolism changes to allow production of a different distribution of materials within the cells. The present results show no evidence of increase in excretory products at photosynthesis-saturating light intensity. If the overall product of photosynthetic metabolism is only in new cells, then under truly steady-state conditions the rate of photosynthesis cannot exceed an equivalent rate of growth.

The second explanation now appears more probable, especially since it has also been demonstrated that overall metabolism in Chlorella is subject to considerable variation (**9**). The CO_2/O_2 quotient of 0.9 often observed for Chlorella in short-time manometric experiments may be compared with the value of 0.76 observed in the present long-time experiments. It is suggested that the quotient of 0.9 is only a transitory value describing a temporary metabolic condition of abnormally high carbohydrate synthesis. After long exposure to high light intensity one might expect a metabolic shift to a synthesis of more reduced materials such as lipids, but this would require a lower oxygen and higher hydrogen content in the cells and is not borne out by the present cellular analyses. A satisfactory description of metabolism under prolonged photosynthesis-saturating intensities will require more complete data on cellular analysis.

Summary

1. The continuous-culture apparatus has been adapted to the study of the carbon balance of *Chlorella pyrenoidosa* during growth by glucose assimilation in the dark. About 49% of the glucose carbon is assimilated and about 93% of the glucose carbon is recovered as carbon dioxide and cellular carbon.

2. A second method has been devised for the study of metabolism in mass cultures of algae. The algal suspension occupies a small portion of a large bottle adapted to a roll culture. Gas exchange is measured by Haldane analysis.

3. The mass culture method has been applied to a study of carbon and nitrogen balance during photosynthetic growth. About 95% of the carbon taken up as CO_2 and the nitrogen taken up as nitrate was recovered as cellular carbon and nitrogen. The recovery was about equally complete under light-limiting or light-saturating intensities for growth.

4. A CO_2/O_2 exchange quotient of 0.70 at low light intensity (50 fc) and with nitrate as the nitrogen source confirms previous measurements by manometric methods. The quotient of 0.76 observed at high light intensity (500 fc) is significantly lower than the value of 0.9 commonly reported from short-time manometric measurements. It is suggested that when Chlorella is first exposed to high light intensity its metabolism temporarily adjusts to an increased carbohydrate synthesis which permits the higher quotient of 0.9 observed in the usual short-time experiments.

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UNIVERSITY OF TEXAS
AUSTIN, TEXAS

LITERATURE CITED

1. BARKER, H. A. Photosynthesis in diatoms. Arch Mikrobiol. **6**: 141–156. 1935.
2. CRAMER, M. and MYERS, J. Nitrate reduction and assimilation in Chlorella. Jour. Gen. Physiol. (in press).
3. KROGH, A., LANGE, E., and SMITH, W. On the organic matter given off by algae. Biochem. Jour. **24**: 1666–1671. 1930.
4. MYERS, J. Culture conditions and the development of the photosynthetic mechanism. III. Influence of light intensity on cellular characteristics of Chlorella. Jour. Gen. Physiol. **29**: 419–427. 1946.
5. ————. Culture conditions and the development of the photosynthetic mechanism. IV. Influence of light intensity on photosynthetic characteristics of Chlorella. Jour. Gen. Physiol. **29**: 429–440. 1946.
6. ————. Culture conditions and the development of the photosynthetic mechanism. V. Influence of the composition of the nutrient medium. Plant Physiol. **22**: 590–597. 1947.
7. ————. Oxidative assimilation in relation to photosynthesis in Chlorella. Jour. Gen. Physiol. **30**: 217–227. 1947.
8. ————, and CLARK, L. B. Culture conditions and the development of the photosynthetic mechanism. II. An apparatus for the continuous culture of Chlorella. Jour. Gen. Physiol. **28**: 103–112. 1944.
9. MYERS, J. and CRAMER, M. Metabolic conditions in Chlorella. Jour. Gen. Physiol. (in press).
10. SHAFFER, P. A. and SOMOGYI, M. Copper-iodometric reagents for sugar determination. Jour. Biol. Chem. **100**: 695–713. 1933.
11. UHVITS, R. The formation of acetaldehyde by *Chlorella pyrenoidosa*. Dissertation, University of Michigan. 1936.
12. WHELTON, R. and DOUDOROFF, M. Assimilation of glucose and related compounds by growing cultures of *Pseudomonas saccharophila*. Jour. Bact. **49**: 177–186. 1945.

THE CHEMICAL COMPOSITION OF CHLORELLA; EFFECT OF ENVIRONMENTAL CONDITIONS

H. A. Spoehr and Harold W. Milner

(WITH FIVE FIGURES)

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Introduction

The purpose of the investigations here described was to determine to what extent the chemical composition of a plant can be altered by growing it under different environmental conditions. The three general processes which determine the organic composition of a plant are photosynthesis, respiration, including aerobic and anaerobic catabolism, and biosynthesis. In the present state of knowledge it is difficult to discern to what extent these three processes are interrelated. For the immediate purposes of this investigation it is, in fact, not essential to attempt to determine which of these processes may be responsible for any observed changes in composition.

If decided changes in composition are observable as results of altered environmental conditions, these effects can then be further analyzed and can perhaps ultimately be ascribed to one or another of the major processes. The immediate problem was to discover which environmental conditions would, when altered, affect the organic composition of the plant, and to determine the nature and magnitude of the effect. The objective was to find environmental conditions which would materially modify the relative proportions of the three major plant constituents, carbohydrates, proteins and lipids.

For such an undertaking, very little information was available as a basis for a working hypothesis to aid in the choice of environmental conditions. It was obvious from the outset, therefore, that a large number of experiments would have to be made.

In an investigation requiring many analyses, the determination of the organic composition of the plants in terms of definite chemical compounds, or even of groups of compounds, would be very laborious and time consuming. Regarding all of the organic carbon of the plant as arising from the reduction of carbon dioxide, we were more concerned with the energy level of this total organic matter than with the isolation of particular constituents. The determination of the heat of combustion of the plant material would, in a measure, satisfy this requirement; but such data would have a limited usefulness. Recourse was taken to a very simple means of determining the "degree of reduction" of the entire organic material of the plant from its elementary chemical composition. This general concept, which has been used but little in connection with problems of this nature,

also permits calculation of the proportions of the major plant constituents and will be discussed in the first section of this paper.

The choice of the plant material for such an investigation is doubtless of great importance. Higher plants are probably not very flexible in respect to their organic composition. They have certain set requirements already imposed by their elaborate structural elements, which constitute a large portion of their total substance. It is conceivable that lower organisms have not such set requirements, and that their degree of flexibility in regard to chemical composition may be greater. The unicellular green algae, for example, grow rapidly and permit relative simplicity in conducting controlled culture experiments. For this reason, and because its extensive use in the study of photosynthesis afforded considerable information concerning its behavior, *Chlorella pyrenoidosa* was used in the present investigations. It was found to be very suitable for such experiments because it grows well under a wide variety of conditions.

It is hoped that the results here described may have some significance in the interpretation of data obtained in photosynthesis experiments. In conjunction with the determinations of the chemical composition of Chlorella cells grown under different conditions, it was originally planned to carry out determinations of the rate of photosynthesis and of respiratory and photosynthetic quotients of cells from the same cultures. Unfortunately, conditions imposed by the war forced postponement of this part of the program, and since then it has not been possible to undertake it.

The Degree of Reduction of Carbon and the R-Value

We are here concerned with the unique ability of the green plant to use carbon dioxide and water as its principal raw materials and to elaborate from the carbon and hydrogen contained in them a host of organic compounds, often molecules of great complexity. The raw materials have a high oxygen content, 72.71% in carbon dioxide and 88.81% in water. The constituents of the plant have a much lower percentage of oxygen. For example, analyses of entire Chlorella cells show a range of oxygen content from 34% to as low as 18%.

It is apparent that the plant has, in the synthesis of its components, eliminated a large part of the oxygen content of the carbon dioxide and water. From a chemical viewpoint this is a process of reduction. An input of energy is required to make the process go, and the reduced compounds which are formed represent a storage of energy. The level of reduction to which the carbon has been carried represents the amount of energy stored. A numerical expression of the level or degree of reduction of carbon and of the energy content of carbon compounds can be derived through the application of simple chemical principles.

The degree of reduction of the carbon in an organic compound is related to the percentage of carbon, hydrogen, and oxygen. Since oxidation

and reduction are the reverse of one another, the most oxidized carbon compound, carbon dioxide, may equally well be spoken of as the least reduced form of carbon. Hence, carbon dioxide has a degree of reduction of zero. The most reduced carbon compound, which represents the highest degree of reduction of carbon, is methane. The degree of reduction of carbon in all other organic compounds will, then, be intermediate between that of carbon dioxide and that of methane.

The degree of reduction of an organic compound can be measured by oxidation. Complete oxidation converts all of the carbon to carbon dioxide and all of the hydrogen to water. The energy released as heat in the combustion is equal to the energy stored by the reduction. The amount of oxygen required to accomplish complete oxidation of an organic compound is a measure of both the degree of reduction and of the energy stored in the compound.

The ratio of oxygen to carbon and to hydrogen for their complete oxidation to carbon dioxide and water is expressed by:

$$\frac{O_2}{C} = \frac{32}{12.01} = 2.664 \text{ and } \frac{O}{H_2} = \frac{16}{2.016} = 7.936$$

The degree of reduction can be expressed by: $(\%\ C \times 2.664) + (\%\ H \times 7.936)$. Starting with the most highly reduced carbon compound, methane, which contains 74.87% carbon and 25.13% hydrogen, we calculate its degree of reduction as:

$$(74.87 \times 2.664) + (25.13 \times 7.936) = 398.9$$

In words, methane combines with 398.9% its own weight of oxygen during complete combustion to carbon dioxide and water.

In the case of a compound containing oxygen, less oxygen will be required for complete oxidation for two reasons. Firstly, because of the presence of the oxygen in the compound, the percentages of carbon and hydrogen are diminished. In the second place, because the compound contains oxygen, the carbon may be considered to be already partially oxidized. Therefore, to find the degree of reduction of a compound containing oxygen, one must calculate how much more oxygen is required to complete the oxidation of the carbon and hydrogen to carbon dioxide and water. This is done by calculating, as before, the total oxygen required, then subtracting the percentage of oxygen already present. Take for an example methanol, which has 37.48% carbon, 12.58% hydrogen and 49.94% oxygen. Its degree of reduction is expressed by:

$$(37.48 \times 2.664) + (12.58 \times 7.936) - 49.94 = 149.8.$$

The remaining element to be considered in the calculation of the degree of reduction is nitrogen. This is regarded as inert. The nitrogen content of the compounds with which we shall deal is predominantly in the form of amino groups. In the complete oxidation of this type of compound,

the nitrogen appears in the elementary form among the products of combustion. Therefore, its effect upon the degree of reduction of the compound is due merely to the fact that its presence diminishes the percentage of carbon and hydrogen in the compound. For example, consider two compounds which differ in empirical formula only by two nitrogen atoms:

Propanol	C_3H_8O	59.96% C,	13.42% H,	26.62% O
Ethyl urea	$C_3H_8ON_2$	48.62% C,	10.88% H,	21.59% O

The degree of reduction of these compounds, calculated as shown for methanol, is: propanol, 239.6 and ethyl urea, 194.3.

THE R-VALUE. The degree of reduction as calculated for the foregoing compounds is an inconvenient number for comparison of values. We have, therefore, used a derived scale to express the R-value. Since carbon dioxide has a reduction level of zero, it is zero on the R-value scale also. Because methane is the most highly reduced carbon compound, it is assigned an R-value of 100. The degree of reduction of any other organic compound can be expressed as its *percentage* of the degree of reduction of methane. This percentage value is from now on referred to as the R-value. The general formula for calculation of R-value is expressed by:

$$\frac{[(\%\ C \times 2.664) + (\%\ H \times 7.936) - \%\ O] \times 100}{398.9} = \text{R-value}$$

So far we have considered specific chemical compounds. The concept of R-value is equally applicable to mixtures of organic compounds, even when the chemical structure of these is unknown. Therein lies its application in the present investigation. We are interested in the total organic material produced during the life of a Chlorella culture; the level of reduction which the carbon of the materials comprising the entire plant has reached; how much energy has been stored in the over-all process.

The four elements, carbon, hydrogen, nitrogen and oxygen add up to nearly 100% in the analysis of the organic material making up a whole plant. The first three are determined by combustion analysis of the plant and are expressed upon an ash-free basis. The difference between 100% and the sum of the percentages of carbon, hydrogen and nitrogen is reported as per cent. oxygen. The R-value of the material is then calculated by the general formula given above.

It is realized that sulfur and phosphorus are also constituents of plant material, that they too affect the oxygen requirement for complete oxidation of the material and so affect the R-value. However, these elements are present in such small percentages that their effect upon the R-value of the plant material is considered negligible, within the range of the over-all experimental errors. If sulphur and phosphorus were determined, their effect would be to increase the reported R-values by a very slight amount. Even one per cent. sulfur, an enormous amount in terms of an entire plant, would raise the calculated R-value by only 0.4 unit. The effect of one per

cent. phosphorus would be still less, since it is probably present as phosphate, already oxidized, and would in fact, largely appear in the ash.

Although its calculation is based upon the degree of reduction of carbon in organic compounds, the R-value is more directly an expression of the energy content of organic materials. It is directly proportional to the heat of combustion *per gram* (THORNTON, **18**). As an energy unit it has greater value in measuring the over-all effect of photosynthetic and metabolic activity than other units which might have been used. A scale of reduction based upon molecular quantities, for instance, is not applicable to material

TABLE I

CHEMICAL COMPOSITION AND R-VALUE OF SOME BIOLOGICALLY IMPORTANT COMPOUNDS

COMPOUND	FORMULA	% C	% H	% N	% O	R-VALUE
Methane	CH_4	74.87	25.13			100.00
Isoprene	C_5H_8	88.16	11.84			82.43
Carotene	$C_{40}H_{56}$	89.48	10.52			80.68
Triolein	$C_{57}H_{104}O_6$	77.32	11.84		10.84	72.48
Chlorophyll a	$C_{55}H_{72}O_5N_4Mg$	73.93	8.12	6.27	8.95	63.28
Leucine	$C_6H_{13}O_2N$	54.93	9.99	10.70	24.40	50.45
Asparagine	$C_4H_8O_3N_2$	36.36	6.10	21.21	36.33	27.31
Ethanol	C_2H_6O	52.14	13.13		34.73	52.23
Glycerol	$C_3H_8O_3$	39.12	8.76		52.12	30.48
Starch	$(C_6H_{10}O_5)_n$	44.44	6.22		49.34	29.70
Glucose	$C_6H_{12}O_6$	40.00	6.71		53.29	26.69
Pyruvic acid	$C_3H_4O_3$	40.91	4.58		54.51	22.75
Malic acid	$C_4H_6O_5$	35.83	4.51		59.66	17.94
Oxalic acid	$C_2H_2O_4$	26.68	2.24		71.08	4.46
Carbon dioxide	CO_2	27.29			72.71	0

which is a mixture of organic substances, some of great complexity and some of unknown structure. Moreover, the elementary analyses from which R-values are calculated give additional chemical information about the materials investigated. The chemical analyses and R-values of some organic compounds of biological importance are shown in table I.

Methods

CULTURE OF CHLORELLA PYRENOIDOSA

All of the cultures herein described were inoculated with descendants from a single slant of *Chlorella pyrenoidosa* (Emerson's strain). This stock was maintained during the course of the experiments by transfer onto fresh agar slants. From a slant the alga was put into liquid culture in test tubes. The test tube cultures were used to inoculate "stock cultures". Stock cultures were grown in 500 ml. flasks fitted with rubber stoppers carrying glass tubes for aeration and transfer. Each experimental culture was inoculated by aseptic transfer of 15 ml. of a stock culture. When it was desired to start a number of experimental cultures with identical inocula, one stock culture served to inoculate as many as 10 larger cultures.

The experimental cultures were grown in Fernbach flasks closed with rubber stoppers. Each stopper carried three glass tubes: a short wide tube with cotton plug, serving as a gas outlet; a short, bent tube for introduction of inoculum; a gas inlet tube reaching to the bottom of the flasks. The tube for inoculation was, of course, plugged with cotton and a cotton-filled filter was attached to the gas inlet tube. The flask containing 2000 ml. of mineral nutrient solution was fitted with stopper, tubes and filter. Cotton batting was wrapped to a thickness of about an inch around the neck of the flask, stopper and tubes and was tied in place. The entire assembly was then sterilized by steam for 20 minutes at 15 pounds pressure.

After the cultures were inoculated, they were allowed to grow for the desired length of time under a given set of environmental conditions. The apparatus used to control these conditions was quite simple. A wooden framework, open on the sides and with a circular opening in the top, formed the support. An incandescent lamp was centrally placed below the top opening. A reflector below and around the lamp directed the light upward. The culture flask rested upon glass rods in a glass dish, which in turn was supported in the circular opening in the top of the framework. The distance from the lamp bulb to the bottom of the culture flask was 20 cm.

The light intensity incident upon the culture was dependent upon the rating of the frosted Mazda lamps. Lamps ranging from 25-watt to 500-watt capacity were used in various experiments. Unless otherwise noted, illumination of the cultures was continuous throughout their period of growth. Water was circulated through the glass dish in which the culture flask stood. The rate of flow of the water served to maintain the culture at any desired temperature.

A gas mixture of predetermined composition was continuously bubbled through the cultures. The agitation caused by bubbling largely prevented settling of the Chlorella cells. In addition, the cultures were shaken by hand several times daily. The gas mixtures were either purchased in cylinders or, in the case of carbon dioxide in air, prepared with use of a compressor unit. The other factor subjected to experimental variation was the composition of the nutrient solution.

The number of days allotted for the growth of each culture varied with the experimental objectives. At the end of the time selected, each culture was examined microscopically for contamination. Very few of the cultures became contaminated. If any organism other than Chlorella was found, the culture was discarded. All analytical data presented in this paper are, therefore, based upon *pure* cultures of Chlorella, not upon merely unialgal cultures.

The cultures found to be pure were quickly cooled to about 2° C. The cells were separated from the medium in a centrifuge. The sedimented cells were resuspended in a little distilled water to rinse off entrained nu-

trient solution, following which they were again centrifuged sharply. The fresh weight of the cells was recorded at this point. The cells were put immediately into a vacuum desiccator to dry at room temperature over calcium chloride. After 24 hours the desiccant was changed and the drying in vacuum was continued until the cells reached constant weight, usually 48 to 72 hours altogether.

Analytical Methods

Since the objective of this work was to discover what changes in chemical composition of an alga could be induced by changes in the environmental conditions under which it grows, it was important to account for all of the organic matter produced. The completely cell-free solutions from several cultures were analyzed for organic matter. The total organic carbon found in the cell-free culture solutions varied from 2.4 to 3.9% of the total carbon content of the cells themselves. From this it is clear that analysis of the cells represents from 96 to 98% of the total organic matter produced by a Chlorella culture. The small amount of organic matter found in solution in the culture medium was not examined further.

The dry cell mass was pulverized in an agate mortar until all of it passed through a 60-mesh screen. A sample was then taken for carbon-hydrogen analysis. Another sample was then ground as fine as possible in the agate mortar and was used for the nitrogen determination.

Samples of Chlorella cells for combustion were handled with the usual precautions applying to hygroscopic materials. Each sample was dried in vacuum over phosphorus pentoxide. Sample and boat were protected in a "piggie" during weighing and transfer.

Nitrogen analyses were made by the micro-Dumas method as described by Pregl.

Carbon and hydrogen were determined by the macro method. The combustion tube filling described by Fisher (5) was used. The samples were burned in platinum boats. The unburned residue from the carbon-hydrogen analysis was reported as per cent. ash.

Complete combustion of material such as Chlorella in the carbon-hydrogen analysis offers some difficulty. Being a mixture of carbohydrate, protein and lipid materials, the sample exhibits a combination of the annoying features of each during combustion. Samples having different compositions behave differently while burning. In general, it is advisable to proceed slowly and to use as low a temperature in the boat-heating unit of the furnace as will insure complete combustion. At first the sample decomposes and tarry substances distill from the boat and condense on the tube. By heating slowly, these deposits can be burned clean. After the decomposition products have left the combustion boat, the carbonaceous residue will ignite. Usually it burns clean, leaving a light colored, fluffy ash. The character of the ash was found to vary with the mineral nutrient

medium in which the sample of Chlorella had grown. High nitrate media yielded Chlorella, the ash from which gave unusual difficulty in the carbon-hydrogen analysis. The melting point of this ash was so low that frequently the ash would fuse during the ignition of the sample. Sometimes carbon was left in this fused ash, and resisted the usual devices for combustion of the last traces. In such cases, the mixture of ash and unburned carbon was weighed, washed with dilute hydrochloric acid, and the carbon determined separately. The weight of this residual carbon was subtracted from the weight of ash plus carbon to get the weight of the ash, and was added to the main carbon determination to obtain the correct carbon content of the sample. Only a few samples required this treatment.

Calculation of the R-value

From the percentages of carbon, hydrogen, nitrogen and ash, determined as described, the R-value of the organic matter produced by each of the Chlorella cultures was calculated. The R-value applies only to the organic constituents of the entire cell mass, hence the percentages of carbon, hydrogen, and nitrogen were calculated to an ash-free basis. The oxygen content of the organic matter was taken as 100% less the sum of the percentages of ash-free carbon, hydrogen and nitrogen. Phosphorus and sulfur were disregarded, as explained in the section on R-value. By use of the general formula given there and the percentages of carbon, hydrogen and oxygen expressed on the ash-free basis, the R-value of the Chlorella was computed.

Composition and R-Value

Dry Weight

Because we are concerned with the quantity and composition of the organic matter produced by Chlorella cultures, all cell yields are expressed as grams dry weight per two liter culture. This is an important point for questions under consideration. Frequently the larger of two yields in fresh weight is the smaller in dry weight, or vice versa. In series of cultures which differed only in the length of time which was allowed for growth, it was sometimes found that the yield in grams fresh weight actually decreased, whereas the yield in grams dry weight from the same cultures showed a steady increase with age. The cell count is also an unreliable index of the weight of organic matter produced, because Chlorella cells vary greatly in size at different times in the life of the culture under some environmental conditions.

The per cent. dry weight of the Chlorella cells obtained from a culture does not enter into the determination of the composition or R-value. This percentage is not without interest, however. Under widely different cultural conditions, the per cent. dry weight of Chlorella was observed to vary from 11 to 42% of the fresh weight. The usual range of this value in

most of the cultures grown was 20 to 33%. The composition and concentration of the mineral nutrients appear to have an effect upon the per cent. dry weight of Chlorella. Under a given set of conditions, the per cent. dry weight tends to increase with the yield of cells and the age of the culture. Cells of high R-value tend to have a high per cent. dry weight. This is probably because they are grown for a long time and because they contain a greater proportion of non-hydrophylic constituents.

Ash

No analyses were made of the composition of the ash of Chlorella. Its percentage was determined in order to calculate the percentages of carbon, hydrogen and nitrogen to an ash-free basis in the computation of R-value. The percentage of inorganic material entering into the composition of Chlorella cells is largely influenced by the proportions and concentrations of the mineral salts in the nutrient medium. To a lesser extent the per cent. ash depends upon the yield of cells produced. The ash content decreases as more organic matter is produced. The range observed in ash content was from 1.5 to 20%. The majority of the cultures produced Chlorella having an ash content of 2 to 10%. The cells of high R-value usually had less than 5% ash.

Carbon and Hydrogen

As would be expected from the way in which R-value is calculated, the carbon and hydrogen content of Chlorella cells bear a linear relationship to the R-value. The small variations from linearity are readily explained in relation to the varying nitrogen content of the samples. The per cent. carbon, on an ash-free basis, varied from 49.5% at the lowest R-value to 70.2% in the sample of highest R-value. The corresponding figures for per cent. hydrogen, ash-free, were 6.78% and 10.53%.

The atomic ratio of hydrogen to carbon was calculated for a number of Chlorella samples. This ratio proved to have little value for our purpose because of the considerable variation in nitrogen content between samples having nearly the same carbon and hydrogen content. The hydrogen to carbon ratio varied only from about 1.6 to 1.8, while the R-value changed from 42 to 55 for the samples compared by both methods.

Nitrogen and Oxygen

The relation of nitrogen content of Chlorella to R-value will be discussed in another section of this paper. The observed range of nitrogen content was from 1.17 to 14.11%. In general, the per cent. nitrogen decreases with increase in R-value, but the relation is not a simple one.

The percentage of oxygen in Chlorella also decreases as the R-value rises. Here again the relation is not linear because of the fluctuation of nitrogen content. If, however, the sum of per cent. nitrogen and per cent.

oxygen is plotted against the R-value, the points lie very close to a straight line. This sum decreases from 43.5 to 19.5% as the R-value increases from the lowest to the highest observed.

R-Value of Chlorella

In these experiments the R-values varied from 38 to 63. The change in R-value during the growth of a culture is continuous rather than stepwise. Three hundred and twenty cultures were analyzed. The distribution of observed R-values between the lowest and the highest is remarkably uniform.

In table II are shown the relation between R-value and elementary

TABLE II

Variation in composition and R-value of chlorella

R-value	Calculated on ash-free basis:				
	% Ash	% C	% H	% N	% O
37.92	3.45	49.51	6.78	9.31	34.40
40.08	3.78	51.00	6.90	11.29	30.81
42.41	20.21	51.65	7.37	14.11	26.87
43.99	5.81	54.59	7.28	10.38	27.75
43.99	8.56	54.79	7.93	5.28	32.00
45.92	7.88	54.58	8.11	10.75	26.56
45.92	2.28	56.01	7.84	7.86	28.29
50.18	2.87	59.73	8.56	4.80	26.91
54.37	1.36	63.77	8.97	3.08	24.18
57.05	5.32	65.35	9.68	1.62	23.35
60.29	4.57	68.18	10.01	1.28	20.53
61.87	3.44	69.19	10.33	1.17	19.31
63.33	3.46	70.17	10.53	1.43	17.87

composition, the range covered by these values, and the differences in composition which may be found in samples of the same R-value.

R-Value of Other Plant Material

The R-values of a number of other plants were determined. Many of these plants had grown under natural conditions. The R-values of the algae analyzed were as follows: *Amphipleura rutilans* 33.76; *Navicula torquatum* 36.22; *Nitzschia closterium* 40.05; *Anabaenopsis* sp. 37.88; *Peridinium cinctum* 41.93; *Ulva* sp. 30.53: *Chlamydomonas* sp. 35.27; *Stichococcus bacillaris* 43.89; *Gigartina Agardhii* 29.85; of the following the blades only were analyzed: *Macrocystis pyrifera* 34.24; *Egregia Menziesii* 36.27.

The dried leaves of several common plants were also analyzed and showed the following R-values: *Zea Mays* 31.88; *Medicago sativa* 38.00; *Helianthus annuus* 38.37; *Linum usitatissimum* 40.08.

That the R-value of leaves may vary with the composition of the leaves

is illustrated by the following. Nasturtium leaves with a starch content of 2% had an R-value of 38.45, and those with a starch content of 20% had an R-value of 34.44. Similarly, tobacco leaves with starch content of 2.5% and 44% had R-values of 38.77 and 34.26 respectively. Obviously, the accumulation of a substance having a low R-value such as starch, results in a decrease of the R-value of the whole leaf.

It is of interest that in the complicated processes comprising the conversion of vegetable material to fuels of higher heating values, there occurs a notable decrease in the oxygen content. This change is accompanied by a marked increase in the carbon content, in the R-value, and hence also in the calorific value, of the material which has undergone this type of conversion. The elementary composition and the R-value of some of these products are given in table III.

TABLE III

COMPOSITION AND R-VALUES OF SOME FUELS

MATERIAL	CALCULATED ON AN ASH-FREE BASIS:				
	% C	% H	% N	% O	R-VALUE
Wood, Basswood	49.05	5.88	0.62	44.45	33.32
" Sugar maple	49.52	5.99	0.69	43.80	34.00
Peat*	55.54	6.28	1.72	36.56	38.18
Lignite*	72.95	5.24	1.31	20.50	53.56
Bituminous Coal*	84.24	5.55	1.52	8.69	64.74
Anthracite Coal*	93.50	2.81	0.97	2.72	67.09
Petroleum	87.94	11.21	0.85		80.82

* Analyses taken from F. W. Clarke, The Data of Geochemistry, U. S. Geological Survey, Bulletin 770, p. 773, 1924.

CONSTITUENTS OF CHLORELLA

The elementary composition and R-value of Chlorella are obtainable with relatively little effort compared to the tedious analysis of plant material for carbohydrate, protein and fat content which are of great interest and importance from a physiological viewpoint. Fortunately it is easy to calculate the approximate lipid, protein and carbohydrate content from the elementary analysis and R-value.

We may take 28 as the R-value of the carbohydrate fraction, a value between that of hexose and of polysaccharide. From elementary analyses of proteins which are available in the literature, an R-value of 42 is obtained. Calculated from published fat analyses, the R-value of plant fats is about 67.5. This value is taken to apply to the total lipid fraction of Chlorella. Per cent. nitrogen multiplied by 6.25 is used as the per cent. protein. With this value as one constant and the R-value of the entire sample as another, algebraic solution of simultaneous equations gives the calculated percentage of carbohydrate and of lipid. Using P for protein,

C for carbohydrate and L for lipid the equations are:

$$(\%\ P \times 42) + (\%\ C \times 28) + (\%\ L \times 67.5) = \text{R-value} \times 100\%$$
$$\%\ P + \%\ C + \%\ L = 100$$

For example take Chlorella with 4.53% nitrogen and R-value 50:

$$4.53 \times 6.25 = 28.3\%\ P.\quad \text{Then, } C + L = 71.7\%$$

and, $(\%\ C \times 28) + (\%\ L \times 67.5) = (50 \times 100\%) - (28.3 \times 42)$. Solving; carbohydrate = 26.2% and lipid = 45.5%.

Calculations made by this method indicate remarkable variations in the proportions of the three major components of Chlorella as the R-value changes. This change is summarized in table IV for the highest, lowest

TABLE IV

CONSTITUENTS OF CHLORELLA CALCULATED FROM R-VALUE

R-VALUE	PROTEIN %	CARBOHYDRATE %	LIPID %
38	58.0	37.5	4.5
42	50.0	32.2	17.7
50	28.3	26.2	45.5
56	15.7	19.0	65.3
63	8.7	5.7	85.6

and intermediate R-values. These figures indicate that there is nearly a linear relation between the increases in R-value and per cent. lipid. The decreases in per cent. carbohydrate and per cent. protein are roughly linear as the R-value increases.

This means of calculating the composition of Chlorella from elementary analysis and R-values has proved to be trustworthy. The calculated percentages of lipid agreed closely with the values which were obtained by solvent extraction. The analyses of Chlorella of different R-values, made by means of solvent extraction, together with an analysis of the fatty acids of Chlorella, will be presented in another paper.

The Effect of Various Environmental Factors

A very considerable amount of work has been devoted to the study of methods of growing Chlorella and similar organisms to be used in investigations of photosynthesis. Valuable contributions to this end have been made through the publications of GAFFRON (**6**), SARGENT (**16**) EMERSON and LEWIS (**3**), PRATT (**11, 12, 13, 14**), TRELEASE and SELSAM (**19**), JACK MYERS (**7, 8, 9, 10**) and others. These investigations have done much to provide what is often regarded as a standard type of organism for photosynthetic studies, though, as MYERS (**7**) points out, "the photosynthetic mechanism has been given an appearance of stability which may not be warranted." The current concepts of the photosynthetic reactions are of necessity derived from observations made under relatively restricted en-

vironmental conditions, during short periods of time, and on the basis of certain assumptions concerning the nature of the products which are formed. We are here concerned less with the photosynthetic phenomena *per se* than with the products which the Chlorella cells are capable of producing under a variety of conditions. That the chemical composition of the cells varies greatly according to the conditions under which they are grown, becomes clearly evident. It need hardly be emphasized, however, that experiments of this type give little direct information as to whether the great variations in composition of the cells reflect differences in the course of the photosynthetic reactions.

In the study of a process as complex as the growth of a Chlorella culture, it is next to impossible to evaluate the effect of one environmental factor operating independently of others. Under the headings which follow, an attempt is made to evaluate the response of Chlorella due principally to variations imposed upon one environmental factor at a time. Generally, the response to variations of a single factor, the effect of which is being studied, depends upon the magnitude of the other factors which are operative.

An important consideration in this type of experimentation is the reproducibility of the results. All cultures were grown in sets of two or more, one culture in each set serving as the control while testing upon the others the effect of an environmental change. Duplicate cultures showed less than 5% difference in yield and less than 1% difference in R-value. Cultures grown under the same set of environmental conditions, but at different dates, showed nearly as good reproducibility. It was also found that a culture with any previously obtained yield and R-value could be grown again at will.

The great magnitude of the observed differences in composition of Chlorella grown under different environmental conditions raises the question, whether such differences in composition are entirely responses to environmental changes or, whether there is involved a selection of a strain of the organism which is adapted to growth under the special conditions. If a particular strain of Chlorella grew selectively under conditions producing high R-values, inoculation of new cultures with these cells of high R-value should give rise to more cells of high R-value. When this was done, the second culture, grown under the same conditions, did not reach as high R-value as the first. A third culture, inoculated with cells from the second, yielded cells of the usual low R-value, even though the three successive cultures all grew under conditions which brought about the production of high R-value cells from the usual inoculum. Also, cells from cultures of high R-value were used to inoculate new cultures which were then grown under conditions favoring the production of low R-value. These cultures yielded cells of low R-value. Apparently, then, the R-value attained by Chlorella depends upon environmental conditions during its growth rather than upon selective growth of a different strain.

Effect of Atmospheric Composition

Although the Chlorella cultures were grown in liquid media, they depended for their source of carbon upon the gas which was bubbled continuously through them. Thus, the composition of the gas stream is an important environmental factor. In most of this work the gas stream was either air or nitrogen, both enriched with 5% CO_2.

Carbon Dioxide. Cultures which depended upon air alone for their source of carbon grew very slowly and produced but one tenth the weight of cells produced by cultures which received air containing 5% CO_2. Comparison of cultures grown with 3% and 5% CO_2 in nitrogen showed no significant difference in yield during ten days at low light intensity, while at high light intensity the yields were roughly proportional to the CO_2 concentration. With 10% CO_2 in nitrogen and various light intensities, the yields were 77 to 87% of those when 5% CO_2 in nitrogen was used, and the R-values of the cultures with 10% CO_2 were also lower. These results are summarized in table V.

TABLE V

Comparative effect of different carbon dioxide concentration on yield and R-value of chlorella

Gas Mixture	Light, watts	Days	Yield, gm/2L.	R-Value
Air	200	15	0.1470	40.44
5% CO_2 + Air	200	15	1.4740	51.44
3% CO_2 + N_2	100	10	0.6617	43.73
5% CO_2 + N_2	100	10	0.6279	43.78
3% CO_2 + N_2	300	12	1.0625	44.18
5% CO_2 + N_2	300	12	1.4408	49.27
5% CO_2 + N_2	100	11	0.9760	45.66
10% CO_2 + N_2	100	11	0.6870	44.39
5% CO_2 + N_2	300	10	1.2508	52.12
10% CO_2 + N_2	300	10	0.9600	46.28

Oxygen. The century-old suggestion of Koene that the primitive atmosphere of the earth contained no free oxygen and that this gas was primarily the result of photosynthetic activity of early forms of plant life, has received considerable attention from geologists and cosmologists (**2, 15, 17**), but very little from plant physiologists. It is impossible to enter here upon a full discussion of this subject. Yet certain aspects thereof are sufficiently germane at least, to warrant their mention. Reference is made more particularly to the formation of coal and petroleum. It has been rather difficult to understand chemically the transformation into coal and petroleum of the constituents of vegetable cells and tissue as we know them today. On the other hand, if under conditions of low oxygen con-

tent of the atmosphere, plants produced compounds of a higher degree of reduction than at present, this may conceivably throw a different light on the problem. In terms of the present investigation, therefore, it is a question whether the presence of oxygen in the cultures of Chlorella affects the R-value of the cells.

It should be mentioned at the outset that our results on the culture of Chlorella with an oxygen-free gas stream in some respects are rather at variance with those of some other investigators. WARBURG (**20**) reported that Chlorella cells are not able to survive in cultures exposed to low partial pressures of oxygen. He ascribed this to nitrite poisoning. EMERSON, STAUFFER and UMBREIT (**4**) also reported that the cells cannot grow when

TABLE VI

THE EFFECT OF THE PARTIAL PRESSURE OF OXYGEN ON THE YIELD AND R-VALUE OF CHLORELLA

GAS MIXTURE 5% CO_2 IN:	LIGHT, WATTS	DAYS	% O	YIELD GM./2L.	R-VALUE
Air	100	11	20	0.7098	45.32
Nitrogen	100	11	trace	0.6912	45.51
Air	200	15	20	1.4740	51.44
Nitrogen	200	15	trace	1.8980	55.21
Oxygen	100	17	95	0.5034	38.67
Oxygen	200	17	95	0.4155	37.92

deprived of oxygen. In contrast with these findings, we obtained excellent growth of cultures when these were supplied with gas mixtures containing only traces or no oxygen, both when fixed nitrogen was supplied in the form of ammonium salts and as potassium nitrate. WARBURG and NEGELEIN (**21**) also found that the rate of photosynthesis of Chlorella decreases when the oxygen pressure is increased from 1/50 to one atmosphere. The experiments here recorded, table VI, show that high concentrations of oxygen result in appreciable differences in both yield and R-value as compared with cultures grown in a gas mixture of carbon dioxide in air or in nitrogen.

A mixture of 5% CO_2 and 95% O_2 was used under different culture conditions. Negligible growth was found in a culture after seven days, daylight illumination, with this gas mixture. Cultures illuminated by 100-watt lamps, one continuously and one intermittently, for 35 days, produced less than half the yield of similar cultures which received 5% CO_2 in air or in nitrogen, and the R-values were lower. These three cultures were grown in a medium containing KNO_3. When the nitrogen was supplied as NH_4Cl, even more striking results were obtained by the use of 5% $CO_2 + O_2$. Two cultures, illuminated by 100-watt and 200-watt lamps

respectively, for 17 days, produced but one third the yield of Chlorella obtained with 5% CO_2 in air or in nitrogen, and the R-values were the lowest ones obtained, 38.67 and 37.92. It is noteworthy that the culture illuminated with a 200-watt lamp produced lower yield and R-value than the culture illuminated with a 100-watt lamp. This is exactly opposite to the effect of higher light intensity upon cultures grown in 5% CO_2 in air or in nitrogen.

A mixture of 5% CO_2 in air contains about 20% oxygen, and the 5% CO_2 in nitrogen which was used contained only a trace of oxygen. Cultures grown for less than two weeks at a low light intensity produced approximately the same yields of cells, of about the same R-value, when either gas mixture was used. Under higher light intensity, the yields were larger and the R-values one to three units higher in cultures grown with the nitrogen mixture than in those which were grown with the air mixture, other conditions being equal. The difference in yield and R-value attained in the two gas mixtures became larger with longer times of growth. This effect was noted both in a medium which produced Chlorella of high R-value and in one which produced large yields of cells of low R-value.

Under intermittent illumination at high intensity, cultures grown with 5% CO_2 in air produced higher yields and R-values than did cultures which received the same exposure to light applied continuously. This was in contrast to the effect noted in the use of 5% CO_2 in nitrogen, as will be discussed more fully in the section on illumination.

Three cultures receiving 5% CO_2 and 95% hydrogen did not differ significantly in yield or R-value from comparable cultures grown with 5% CO_2 in nitrogen.

The effect of using a completely oxygen-free gas stream was tried by passing the 5% CO_2 in nitrogen through a hot combustion tube filled with copper before the gas was bubbled through the culture. Cultures grown under both continuous and intermittent illumination at low light intensity did not differ significantly in yield or R-value from the control cultures which grew with the untreated 5% CO_2 in nitrogen. Under the conditions used, the removal of the trace of oxygen from the gas mixture did not affect the growth of the culture.

Mineral Nutrients

In the course of these experiments a number of variations in the mineral nutrient solution were used. The total salt concentration as well as the proportions of the several ions in solution were varied. The objective was to find solutions suitable for growing cells of high R-value.

Table VII shows the molar concentrations of salts in the six media which were used for most of the experimental cultures. The essential difference between these media is the form in which fixed nitrogen was supplied. Those with nitrate are designated A, ammonium chloride B, and

ammonium phosphate C. The concentration of fixed nitrogen was the same in all these media except in A–2.

Each of the media had a pH of 5.9 when prepared. During growth, the cultures in A media became alkaline, as much as pH 7.2, whereas those in B media became acid, reaching pH 3.5 in old cultures. The change in pH was very small in C media.

It was found that the yield and R-value attained by a Chlorella culture was not determined by the medium alone. In general, cultures grown

TABLE VII

MOLAR CONCENTRATION OF SALTS IN CULTURE MEDIA

	A	A–2	B	B–2	C	C–2
KNO_3	0.00225	0.0250				
NH_4Cl			0.00225	0.00225		
$(NH_4)_2HPO_4$					0.000825	0.000825
$(NH_4)_2HPO_4$					0.000715	0.000715
KCl				0.0300		0.0300
$MgSO_4$	0.0100	0.0200	0.0100	0.0100	0.0100	0.0100
KH_2PO_4	0.0100	0.0180	0.0100	0.0100	0.0100	0.0100
[Fe]	0.000005	0.000005	0.000005	0.000005	0.000005	0.000005

in A–2 medium produced large yields of Chlorella having a relatively low R-value. B and C media were approximately equal in effect upon yield and R-value of cultures grown for short or intermediate times. For a long time of growth at high light intensity the C media were superior to the B in producing larger yields and higher R-values.

WATER. The local tap water supply comes from wells, and has a fairly high mineral content. Before using this water for preparation of culture media, it was boiled vigorously for 20 minutes to drive off the free chlorine. After it had cooled, the considerable quantities of carbonates which had been precipitated were removed by filtration. Nutrient solutions were then prepared by dissolving the mineral salts in this water. Micro-nutrients were not added, as the yields of Chlorella indicated that the water was adequate for providing micro-elements.

A comparison was made between tap water (treated as described) and distilled water, redistilled in all-glass apparatus. The salts of C–2 medium were dissolved in each water and the two cultures grew for 15 days, illuminated continuously with 200-watt lamps. The culture in the medium prepared with doubly distilled water produced only one-sixth the yield and had an R-value seven units lower than the other culture. All cultures here reported were grown in media prepared with tap water, treated as described above.

FIXED NITROGEN. While the R-value of Chlorella can be varied experimentally by imposing a change in any one of several environmental conditions, the extent of the variation in R-value is limited primarily by one

factor. This key factor which determines whether a culture will reach a high R-value is the supply of fixed nitrogen in the medium.

The fixed nitrogen content of the medium for each culture was computed from the nitrogen content of the salts added. From the weight of Chlorella produced and the per cent. nitrogen in the cells, the total nitrogen content of each crop of Chlorella was calculated. The fixed nitrogen remaining in the medium at the time the cells were harvested was estimated as the difference between the original fixed nitrogen and the nitrogen content of the Chlorella cells. This quantity is termed residual fixed nitrogen.

When the residual fixed nitrogen is plotted against R-value, certain relations become clear. No culture reached an R-value higher than 47 when the residual fixed nitrogen concentration was greater than 0.001 molar. Below 0.001 M, the R-value appears to be controlled by environmental factors other than nitrogen concentration. A number of cultures grown under low light intensity and for relatively short time reached residual fixed nitrogen concentrations between 0.001 M and zero, without attaining R-values as high as 47. With higher light intensity and long enough time, all of our cultures in which the residual fixed nitrogen was less then 0.001 M reached high R-values. For R-values of 57 and higher, the residual fixed nitrogen concentrations were below 0.0004 M. With the highest favorable light intensities, and even very long periods of time, if the residual fixed nitrogen concentration was greater than 0.001 M, the R-value remained below 47. Therefore, success in growing Chlorella of high R-value depends primarily upon the fixed nitrogen concentration of the medium.

The fact that an exiguous supply of nitrogen nutrients results in increased fat production in diatoms was observed by BEIJERINCK (**1**). Although these observations were based only upon microscopic examination, they have been entirely confirmed, in principle, by the methods of analysis and with the organism we have used.

If the medium initially contains so much fixed nitrogen that Chlorella cells growing in it do not reduce the concentration below 0.001 M, a culture of high R-value will not be obtained. In these experiments, a high R-value was never found for Chlorella grown in a medium containing 0.025 M KNO_3. When a culture is started with a fixed nitrogen concentration of 0.001 M or less, cells of high R-value are obtained but the yields are extremely small. Larger yields of cells can be obtained by supplying an adequate quantity of fixed nitrogen. With growth of the cells, the fixed nitrogen concentration decreases below 0.001 M and high R-values are obtained. We empirically selected an initial nitrogen concentration of 0.00225 M as suitable for this purpose. Concentrations of ammonium nitrogen much larger than 0.00225 M appeared to have an unfavorable effect upon the growth of Chlorella. Probably, by adjustment of other factors, a somewhat higher concentration of nitrate nitrogen would still permit pro-

duction of cells of high R-value, but only after inconveniently long periods of growth.

Cultures grown in medium A, B and C, all with the same fixed nitrogen concentration, produce about the same yields and R-values under low intensity illumination and relatively short periods of growth. With high light intensity and longer time, the yield increases more rapidly in A medium and the R-value shows more rapid increase in the B and C medium. Finally, after a long time at high light intensity, cultures in the three media approach equality both in yield and in R-value. The greater length of time required for attainment of high R-value in KNO_3 medium makes it less desirable than media containing ammonium nitrogen for the production of Chlorella of high R-value.

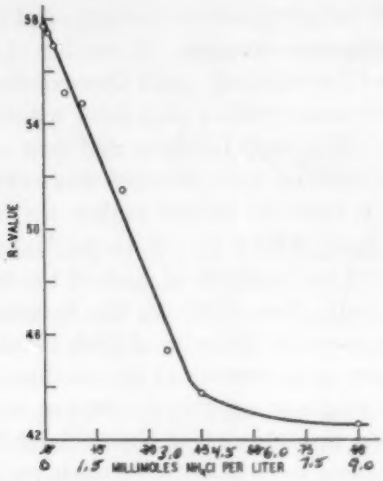

FIG. 1. Effect of nitrogen concentration in culture medium on the R-value of Chlorella.

In the presence of an abundant nitrogen supply, A–2 medium, Chlorella cells remained dark green for as long as we have maintained a culture, 112 days. Cell division ceases after about two weeks. After that time the cell count of a culture does not change, but the weight of the cells may increase by a factor of four to five. This increase in weight is accompanied by a slow increase in R-value from about 42 to about 47, and an increase in cell size which is readily apparent under the microscope.

Entirely different, both in visual appearance and chemical composition, is the course of development of a culture in a medium limited in fixed nitrogen. In the early stages of growth, the appearance, yield and R-value of the cells approximate those of cultures in A–2 medium. When cell division stops, either from age or depletion of the supply of fixed nitrogen, the R-value rises sharply and the appearance of the cells changes. The dark green color becomes lighter, gradually changing to yellow in the cultures of highest R-value. These pale Chlorella cells of high R-value were many

times the size of the dark green cells of low R. The pigment changes will be described in a following section.

The fixation of molecular nitrogen by green algae is a controversial subject. We have no critical evidence for or against nitrogen fixation, but have observed that in a few cultures of high R-value the nitrogen content of the cells was substantially more than the fixed nitrogen supplied to the cultures. This suggests the possibility that Chlorella may, under the special conditions producing high R-value, fix atmospheric nitrogen. Valid evidence in support of this view could be obtained only by much more rigorous control of conditions and refinement of analysis than was used in the experiments described here.

Figure 1 shows the effect of fixed nitrogen concentration in the medium upon the R-value of the Chlorella cells. Series of cultures in B medium

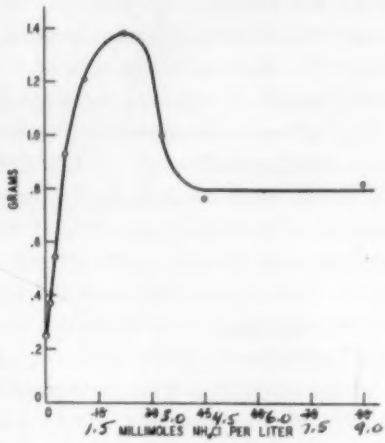

FIG. 2. Effect of nitrogen concentration in culture medium on the yield of Chlorella.

modified to contain varying initial nitrogen content were grown for 15 days under illumination with 200-watt lamps. The fixed nitrogen concentrations tested were 0 to 0.009 M. The yield in grams for the same cultures is shown in figure 2. The maximum yield was obtained with an initial fixed nitrogen concentration of about 0.00225 M. On either side of this concentration the yield decreased. As the nitrogen is reduced from 0.00337 M to almost zero, the R-value increases sharply. Later it was found that by increasing the time of growth of cultures as the nitrogen concentrations were increased from near zero to 0.00225, just as high R-values were obtained at any of these nitrogen concentrations, and that the yield was roughly proportional to the nitrogen concentration. This was not the case with nitrogen concentrations higher than 0.00225. Similar results were obtained from experiments in which different light intensities and different lengths of time were used.

When all the salts of the B medium were varied in the same proportion,

the effect on R-value of the cultures was not significantly different from that of varying the NH_4Cl alone. An increase in all of the salts was reflected in a small increase in yield.

Magnesium. Trelease and Selsam (19) have shown that although Chlorella exhibits a tolerance to high concentrations of magnesium, the yield of cells is small. In order to test the effect of lower concentrations of magnesium, a series of cultures was grown in C–2 medium and modifications in which the magnesium concentration was reduced to 0.005, 0.0025 and 0.001 M with the addition of corresponding quantities of Na_2SO_4 to maintain the same $[SO_4]$ in all. Only slightly higher yields with approximately the same R-value were produced in the three media with reduced magnesium. A similar series of cultures was grown in B–2 medium and modifications with 0.005, 0.0025 and 0.001 M magnesium, the $[SO_4]$ being maintained constant by the addition of K_2SO_4. In this series the yield and R-value decreased with decreasing magnesium. When $MgSO_4$ was omitted from the medium no discernable growth of Chlorella occurred.

Phosphate. Several series of cultures were grown in modifications of the C-medium containing phosphate concentrations from 0.00125 to 0.04 M. The yield and R-values of these cultures did not differ significantly from those of the controls which contained 0.01 M phosphate. A culture to which no KH_2PO_4 was added produced only 2% of the yield of the control.

Potassium. The reduction of [K] from 0.01 to 0.001 M was accompanied by a decrease of 10% in yield and of 2.5 units in R-value. The effect of increasing [K] was much more striking. Series of cultures were grown in both B and C medium to which KCl was added to give 2, 3, 4, 6 and 8 times the [K] in the controls. The yields and R-values showed progressive increases with 2, 3 and 4 times [K]. With 6 and 8 times [K], these values decreased slightly from those obtained with 4 times [K].

The yield and R-values obtained from C medium with added [K] were higher than those obtained from B medium with corresponding additions of [K]. The C medium with 4 times [K] is designated C–2. This medium yielded the highest R-values obtained.

Sodium. When NaCl was substituted in equimolar quantities for additions of KCl as described under the section on potassium, similar effects on yield and R-value were observed. With large additions of NaCl to C medium, 0.13, 0.18 and 0.23 M, the yields and R-values decreased to approximately 70% of the maximum.

Total Salt Concentration. It became apparent that the dilute media frequently recommended for the culture of algae are not suitable for growing the heaviest yields of Chlorella. Beijerinck's, Knop's and Molisch's solutions, for example, have total salt concentrations of about 0.02 M and often are used in diluted form. The media which yielded our best crops of Chlorella had total salt concentrations of 0.052 and 0.063 M (C–2 and A–2).

IRON. The quantity of this element present in the water supply and as impurity in the nutrient salts added was probably sufficient for the needs of Chlorella. No significant difference was observed between cultures without $FeCl_3$ added and those with additions up to 0.00001 M.

ILLUMINATION

INTENSITY. When the other environmental conditions were favorable, cultures grew well under light intensities ranging from that provided by a 25-watt lamp to full sunlight. With 25-, 40-, 60-, 75-, and 100-watt lamps the yields in any one medium were approximately proportional to the light intensity. Increase in light intensity above 100 watts caused relatively smaller increases in yield. Under continuous illumination the maximum yields in B, B–2, C and C–2 media were obtained with 200-watt lamps, in medium A–2 with 300-watt lamps. Higher intensity, from a 500-watt lamp, caused a small drop in yield from the maximum. Cultures in 5-gallon bottles, A–2 medium, grown under natural illumination gave the largest yields when exposed to direct sunlight. The effect of light intensity upon the yield and R-value of a culture can hardly be evaluated without consideration of the time factor. The effect of various times of growth at different light intensities is discussed later.

INTERMITTENT ILLUMINATION. Under this heading are included cultures which received illumination from Mazda lamps controlled by a time switch. In the latter, the lights were on for 12 hours and off for 12 hours alternately.

When the gas stream was 5% CO_2 in nitrogen, the yields of Chlorella were very nearly proportional to the total time of illumination. That is, an intermittently illuminated culture produced about half the yield of one illuminated continuously for the same number of days. Also, the yields and R-values were nearly equal for an intermittently illuminated culture and one which received the same total time of illumination continuously.

A different effect was found when 5% CO_2 in air was used. Intermittently illuminated cultures produced 8 – 25% higher yields of cells with R-values 2–3 units higher than did companion cultures which received the same amount of illumination continuously. In this experiment eight cultures were started in the same medium at the same time, supplied with 5% CO_2 in air and illuminated by 200-watt lamps. The continuously illuminated cultures were harvested after 35, 49, 63, and 77 days; the intermittently illuminated ones after 70, 98, 126 and 154 days.

To summarize the conditions producing cells of the highest R-value: C–2 medium was superior to the others tested, 5% CO_2 in nitrogen was the most favorable gas mixture, and 200-watt continuous illumination was the best of those tested.

Groups of experiments were arranged so that one of a pair of intermittently illuminated cultures was harvested at the end of a light period, while the companion culture was harvested at the end of a dark period, each of

the pair having been exposed to the same number of periods of illumination. In each case, the yield was a little less, and the R-value a little higher for the culture harvested at the end of a dark period. These results indicated that it might be possible to increase the R-value of Chlorella by allowing the culture to stand for a longer time in the dark. Some cultures were allowed to stand in the dark up to sixteen days. In none of these cultures was the increase in R-value much over one unit and there was also a considerable loss in weight.

Temperature

In attempting to determine the effect of temperature on the yield of Chlorella cells, the results become complicated by the difference of behavior of the organism in different media. Moreover, light intensity appears to influence the response to temperature of the cultures growing in the same medium. The temperatures employed for these tests ranged from 10 to 40° by 5° intervals.

Cultures in B–2 medium produced the best yields of Chlorella at 20°. There was no visible growth at 30° and low light intensity, but at high light intensity moderately good growth was observed at 30°. Growth was very slow at 10° with either light intensity. The R-value was lower at 10° than at 20°, but did not show more than 2 units variation at temperatures from 15 to 30°.

Chlorella grown in A–2 medium and under high light intensity, produced the greatest yields at 25°, and as good yields at 30° as at 20°. At very high light intensity some growth took place at 35° but none at 40°. While no growth took place in a culture maintained constantly at 40°, this temperature may be exceeded for short times without harmful effects. This was demonstrated in connection with cultures grown for the large scale production of Chlorella in 5-gallon bottles in a greenhouse exposed to direct sunlight. Occasionally the temperature within these cultures was observed to reach 43°. No harmful effect was apparent, judged by subsequent growth of the cultures and by comparison of their yields with similar cultures which had not become as warm. On the other hand, exposure to 40° for only an hour killed the cells of Chlorella cultures which were growing in B medium at low light intensity.

The effect of growing cultures at 15° and 20° with intermittent illumination was not significantly different from that observed at the same temperatures with continuous light.

Storage of energy

In considering the conditions favorable to the production of Chlorella cells of a given composition, an incomplete judgment will be reached if attention is paid to yield alone, or to R-value alone. Because each of these quantities is variable over a large range, both should be considered in the evaluation of the productivity of a culture grown under a given set of en-

vironmental factors. Since the R-value is proportional to the heat of combustion per gram of material, the product of R-value times the yield in grams expresses the total energy stored by a Chlorella culture. This energy unit, the product of R-value and grams yield, is abbreviated to RG.

Of interest from the physiological viewpoint is the extent to which a culture can make use of the light energy available to it. At the beginning of these experiments it was planned to make measurements of the photosynthesis and respiration of Chlorella grown under conditions leading to the production of cells of different R-values. Wartime changes in staff necessitated abandonment of this phase of the investigation. Lacking di-

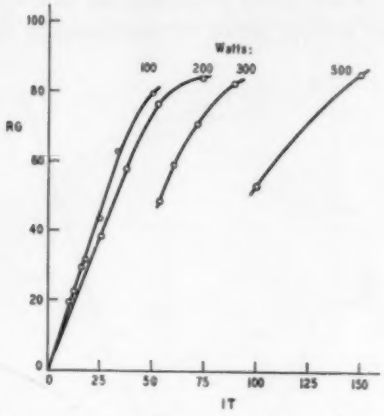

FIG. 3. Relation of RG to IT of Chlorella cultures in B medium under different light intensities.

rect physiological measurements, it becomes important to examine the available data for indications of possible differences in the utilization of light by cultures under various conditons.

We have no measure of the fracton of the incident light which was absorbed by the Chlorella cultures, although it was evident to the eye that this varied from near total absorption to almost complete transmission. We can, however, compare results obtained by growing Chlorella under different intensities of incident illumination. As we are concerned with the measurable over-all effect which has taken place during the entire period the culture was grown, a consideration of light intensity is meaningless without reference to the time factor. The manufacturer's rating of the initial lumens of the lamps served as the intensity factor, I. The time factor is expressed in days. The light energy incident upon a culture is proportional to the product of lumens times days. For convenience, this product is divided by 1000, and is denoted by the abbreviation IT.

When cultures are grown in the same medium and at the same light intensity, the value of RG is proportional to the value of IT over a considerable range. This is shown in figure 3. There is a deviation from the linear relationship as the cultures grow older, because the rate of increase

of both yield and R-value slows up. The curves lie quite close together when the intensity factor of IT is not greater than that which favors the greatest rate of growth of Chlorella in the given medium. For cultures in B medium, nearly the same RG is obtained at low values of IT with 25 to 200-watt lamps. Naturally, the same IT value will be reached in shorter time with higher light intensity. A 200-watt lamp is to be preferred for the attainment of maximum RG value of a culture growing in B medium. If the light intensity factor is greater than that which gives maximum growth, a larger value of IT is needed to produce a given value of RG, as shown by the curves for 300-watt and 500-watt lamps in figure 3.

The value of RG is not quite proportional to IT at very low values of the latter. While the weight factor of the quantity RG increases from the

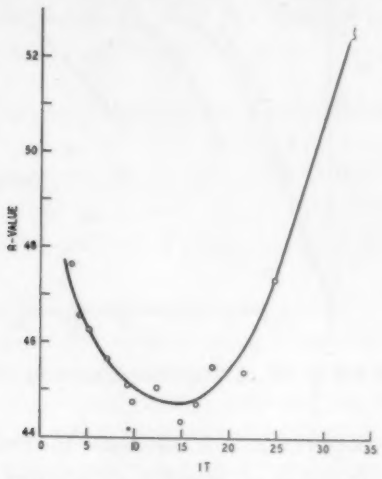

FIG. 4. R-value of Chlorella grown under low light intensity for relatively short times.

start with increase in IT, the R-value actually decreases at first, passes through a minimum at about IT = 15, then starts its steady increase at higher IT values. This is shown on a larger scale in figure 4. The points shown in this dip in the R-value curve are values from cultures grown with lamps of 100 watts and less for periods of 6 to 15 days. For example, an IT value of 15 is reached with a 100-watt lamp in 9.1 days, with 60-watt in 18 days, with 40-watt in 32 days, and with 25-watt in 55 days. With light intensities higher than that from a 100-watt lamp, the minimum R-value would probably be reached in a very much shorter time. Since high R-values were not attained with an IT value of less than 50, it is seen that it would require an inordinately long time to grow cultures of high R-value by use of lamps of small wattage.

The storage of energy as expressed by RG for a given value of IT is also influenced by the composition of the culture medium. This is illustrated by the curves in figure 5, showing the R-value, yield (grams) and RG for cul-

tures grown in media A–2 and C–2, each under the light intensity giving the best yields. This was 200 watts for C–2 and 300 watts for A–2. It is clear that the cells grown in C–2 medium exceed the others in both R-value and yield; hence the RG value is also higher. On the other hand, cells grown in A–2 medium, while producing relatively high yields, have a low R-value and consequently their RG is well below that of the cells grown in C–2 medium. In other words, when both are growing under the most favor-

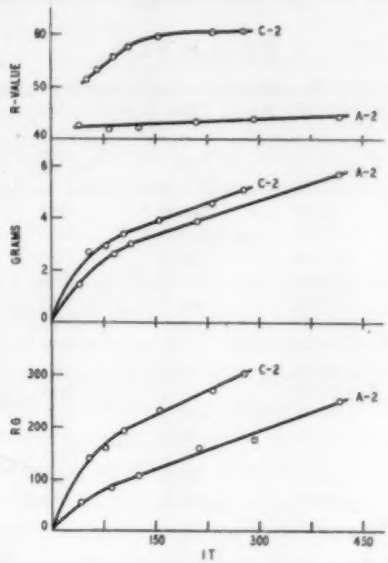

FIG. 5. Differences in the R-value, yield and RG of Chlorella cultures grown for long times at high light intensity in different media.

able conditions, the culture of high R-value stores considerably more energy than does the one of low R-value. Chlorella cells growing under conditions which produce high R-value appear to make more effective use of the incident light than cells grown under conditions producing low R-values.

One of the most striking effects noted in the use of different media was the unusually high yield of Chlorella obtained from A–2 medium at very low light intensity. In 15 days of continuous illumination with a 25-watt lamp, IT = 4, a culture in A–2 medium yielded 0.9 g. of cells. Under similar conditions, cultures in the other media yielded but 0.3 g.

The choice of a medium for Chlorella culture can be based upon two considerations, the chemical composition of cells which is desired, and the yield which is obtained for a given light exposure, the ratio of RG/IT. It is readily apparent that medium C–2 is definitely superior to A–2 for the production of large yields of Chlorella of high R-value. Medium A–2 is the best of the six if it is desired to grow the maximum amount of Chlorella at low light input, or if it is desired to obtain large quantities of cells having a low R-value.

In table VIII are shown high RG values attained by cultures in the various media. The highest RG values were obtained in media C–2 and A–2. It is probable that these values could have been increased if the experiments had been run for a longer time, that is, with higher IT.

Compared to the long continuing increase in RG with increase in IT obtained in C–2 and A–2 media, cultures in other media showed very little increase in RG, some after IT had reached 110, others after IT was only 55. Therefore, it was not profitable to grow cultures in the latter media

TABLE VIII

HIGHEST RG OBTAINED WITH DIFFERENT MEDIA.
(100 WATT, 1650 LUMENS; 200 WATT, 3700 LUMENS; 300 WATT, 5950 LUMENS.)

RG	R-VALUE	GM./2L.	IT	WATTS	DAYS	MEDIUM
305.6	59.56	5.1600	305.6	200	75	C–2
252.4	44.26	5.7020	416.5	300	70	A–2
192.4	56.43	3.4100	103.6	200	28	C–2
136.7	44.26	3.0876	111.0	200	30	A–2
126.5	63.33	1.9980	104.0	100	63	C–2, ¼ N
126.0	56.24	2.2408	111.0	200	30	A
106.0	55.24	1.9192	111.0	200	30	B–2
112.1	42.51	2.6376	55.5	200	15	A–2
99.1	55.32	1.7920	55.5	200	15	C–2
93.9	61.59	1.5242	46.2	100	28	C–2, ¼ N
87.5	51.09	1.7130	55.5	200	15	A
84.8	54.39	1.5594	55.5	200	15	B–2
83.0	53.76	1.5444	55.5	200	15	B
78.1	52.28	1.4939	55.5	200	15	C

beyond these IT values. For comparison with media in the lower IT groups, results obtained with media C–2 and A–2 are included. It is evident that at the lower IT levels also, the C–2 and A–2 media produce the highest RG values. In this table are included cultures in C–2 medium containing ¼ the specified quantity of fixed nitrogen, designated C–2, ¼ N.

PIGMENTS

The color change from dark green to yellow-green and finally to yellow, which accompanies the increase in R-value of Chlorella is a very striking phenomenon. Although this color change was not followed through from the dark green to the yellow cells, pigment analyses were made on selected cultures by Dr. H. H. Strain of this laboratory.

The chlorophyll and carotene content of Chlorella both decrease as the R-value of the cells increases. Among Chlorella cultures of low R-value the chlorophyll content may vary ten-fold, depending upon the culture medium, light intensity and other conditions. This variation, however, is small in comparison to the difference in chlorophyll content between cultures of low R-value and of high R-value. In the latter, the chlorophyll content was only about 1/500, and in extreme cases 1/2000 that of the 6% by weight of chlorophyll found in cells of low R-value.

What is more striking is the relationship between chlorophyll content and cell yield in cultures which have reached high R-values. As their R-value increases, there is a regular decrease in chlorophyll content to about 0.012%. From this point, there is comparatively little change in the chlorophyll content; yet the weight of the cells may increase three-fold, with a corresponding decrease in percentage of chlorophyll.

The decrease in carotene content is about one-tenth as great as the change in chlorophyll content. Obviously this results in a change of the ratio of chlorophyll to carotene.

It appears possible that, at high R-values, the increase in cell yield may be independent of the chlorophyll concentration. This possibility, and other problems relating to the pigment content and increase in organic matter of cells of high R-value, together with their photosynthetic and respiratory activities, should be given more thorough investigation.

Summary

A method is described for determining the degree of reduction of the total organic matter of plant material from its elementary chemical composition. The degree of reduction is designated the R-value; it is proportional to the heat of combustion and is an expression of the energy content of the material. From the elementary analysis and R-value, it is possible to calculate the approximate carbohydrate, protein and lipid content of the plant material. It is shown that these components in Chlorella vary widely with different environmental conditions under which the cells are grown. For example, the lipid content varied from 4.5 to 85.6%.

The influence of various environmental factors on the chemical composition of Chlorella is described. These factors include carbon dioxide concentration; aerobic and anaerobic atmospheres; mineral nutrients, more particularly fixed nitrogen; illumination and temperature. Conditions were found which favor the production of cells containing a large amount of lipid, that is, cells of high R-value. In general, cells having low R-value are produced when the fixed nitrogen in the medium is above 0.001 M; below this concentration, cells of higher R-value can be obtained. High light intensity also favors the production of cells of high R-value. The interrelationship of nitrogen supply and illumination is described. The energy stored by cultures under different environmental conditions is considered in relation to light intensity and time.

Very striking changes in chlorophyll content of Chlorella cells occur with increase in their R-value, indicating that cells with high lipid content carry on photosynthesis with a chlorophyll content 1/500 to 1/2000 that of cells of low lipid content.

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CARNEGIE INSTITUTION OF WASHINGTON,
DIVISION OF PLANT BIOLOGY,
STANFORD, CALIFORNIA

LITERATURE CITED

1. Beijerinck, M. W. Verzamelde Geschriften, pp. 239–241, Delft 1921.
2. Clarke, F. G. The Data of Geochemistry. U. S. Geological Survey, Bulletin 770, pp. 58–61. 1924.
3. Emerson, R. and Lewis, C. M. Factors influencing the efficiency of photosynthesis. Amer. Jour. Bot. **26**: 808–822. 1939.
4. Emerson, R. L., Stauffer, J. F. and Umbreit, W. W. Relationships between phosphorylation and photosynthesis in Chlorella. Amer. Jour. Bot. **31**: 107–120. 1944.
5. Fisher, H. L. Laboratory Manual of Organic Chemistry. New York, John Wiley and Sons. 1920.
6. Gaffron, H. Methoden zur Untersuchung der Kohlensäureassimilation. Abderhalden's Handbuch der Biologischen Arbeitsmethoden **11**: Teil 4: 101–160. 1929.
7. Myers, Jack. Growth of *Chlorella Pyrenoidosa* under various culture conditions. Plant Physiol. **19**: 579–589. 1944.
8. ———. Culture conditions and the development of the photosynthetic mechanism. III. Influence of light intensity on cellular characteristics of Chlorella. Jour. Gen. Physiol. **29**: 419–427. 1946.
9. ———. Culture conditions and the development of the photosynthetic mechanism. IV. Influence of light intensity on photosynthetic characteristics of Chlorella. Jour. Gen. Physiol. **29**: 429–440. 1946.
10. ———. Culture conditions and the development of the photosynthetic mechanism. V. Influence of the composition of the nutrient medium. Plant Physiol. **22**: 590–597. 1947.
11. Pratt, R. Studies on *Chlorella vulgaris*. IV. Influence of the molecular proportions of KNO_3, KH_2PO_4, and $MgSO_4$, in the nutrient solution on the growth of Chlorella. Amer. Jour. Bot. **28**: 492–497. 1941.
12. ———. Studies on *Chlorella vulgaris*. VII. Influence of age of the culture on the rates of photosynthesis and respiration. Amer. Jour. Bot. **30**: 404–408. 1943.
13. ———. Studies on *Chlorella vulgaris*. VIII. Influence on photosynthesis of prolonged exposure to sodium bicarbonate and potassium bicarbonate. Amer. Jour. Bot. **30**: 626–629. 1943.
14. ———, and Fong, F. Studies on *Chlorella vulgaris*. II. Further evidence that Chlorella cells form a growth-inhibiting substance. Amer. Jour. Bot. **27**: 431–436. 1940.
15. Russell, H. N. The Solar System and its Origin. P. 61. Macmillan Co. 1935.
16. Sargent, M. C. Effect of light intensity on the development of the photosynthetic mechanism. Plant Physiol. **15**: 275–290. 1940.

17. SCHUERT, C. The Evolution of the Earth and its Inhabitants. P. 50. Yale University Press. 1923.
18. THORNTON, W. M. The relation of oxygen to the heat of combustion of organic compounds. Phil. Mag. **33**: 196–203. 1917.
19. TRELEASE, S. F. and SELSAM, M. E. Influence of calcium and magnesium on the growth of Chlorella. Amer. Jour. Bot. **26**: 339–341. 1939.
20. WARBURG, O. Über die Geschwindigkeit der photochemischen Kohlensäurezersetzung in lebenden Zellen. II. Biochem. Zeitschr. **103**: 188–217. 1920.
21. ————, and NEGELEIN, E. Über die Reduktion der Salpetersäure in grünen Zellen. Biochem. Zeitschr. **110**: 66–115. 1920.

The following germane references which were not available, because of war conditions, at the time this paper was written, were added to the galley proof:

HARDER, R. and v. WITSCH, H. Bericht über Versuche zur Fettsynthese mittels autotropher Mikroorganismen. Forschungsdienst, Organ der deutschen Landwirtschaftswissenschaft. Sonderheft 16. 1942.

————. Über Massenkultur von Diatomeen. Ber. deutsch. Bot. Ges. **60**: (146)–(152). 1943.

v. WITSCH, H. Beobachtungen zur Physiologie des Wachstums von *Chlorella* in Massenkulturen. Biolog. Zentralb. **67**: 95–100. 1948.

————. Physiologischer Zustand und Wachstums-intensität bei *Chlorella*. Archiv für Mikrobiologie. **14**: 128–141. 1948.

THE EFFECT ON THE GROWTH OF PEANUTS OF NUTRIENT DEFICIENCIES IN THE ROOT AND THE PEGGING ZONE

HENRY C. HARRIS

(WITH TWO FIGURES)

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Introduction

The peanut plant produces flowers above ground, and the flowers when fertilized form gynophores (fig. 1) or pegs. The pegs then push into the soil where they develop into fruit (fig. 2). For this reason both the roots and the fruit of the plant grow in the soil.

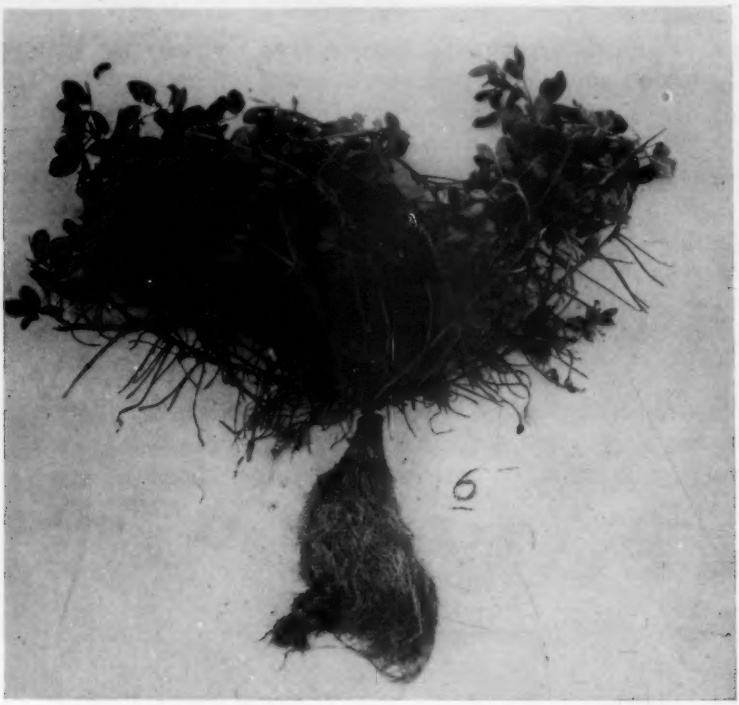

FIG. 1. Peanut plant which received a complete nutrient solution in the root zone and everything except calcium in the pegging zone. Note the abundance of gynophores extending downward from the branches, but developing into very few fruits.

Several authors (**11, 14, 17**) have found root-hair-like growths on the pegs of peanuts. Growths of that nature also have been observed by the writer. Some varieties are known to have similar or root-like formations (**14, 17**) on the hull of the fruit. PETTIT (**11**) as early as 1895 reported

evidence indicating that the gynophores may absorb water and nutrients. VAN DER VOLK (16) found that the gynophores would develop fruit in a soil extract but not in rain water, and WALDRON (17) suggested the possibility of absorption through the pegs. SHIBUYA (12) apparently felt that mineral nutrients in contact with the pegs were not necessary for fructification. More recent evidence (3, 4, 5, 6, 7, 8) as well as some unpub-

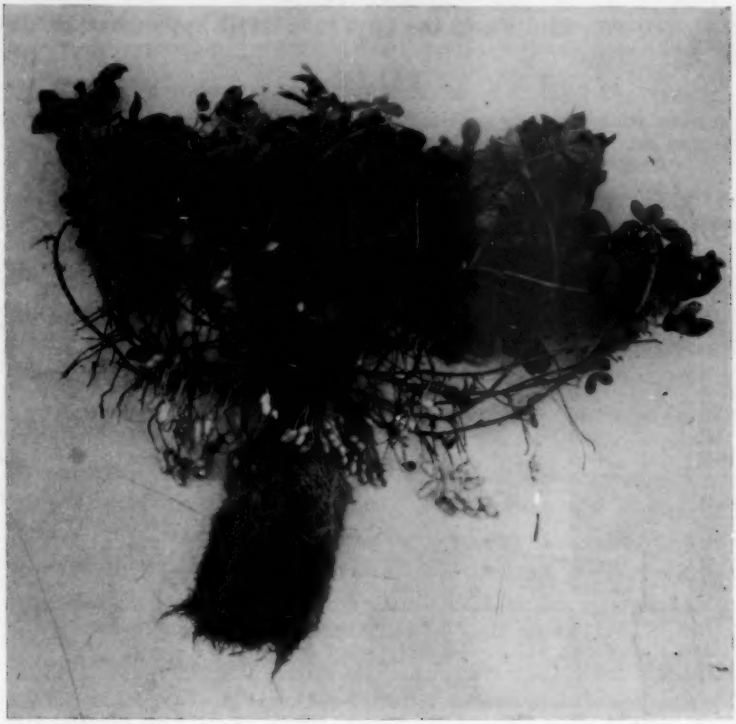

FIG. 2. Peanut plant which received a complete nutrient solution in both the root and pegging zone. Note that many of the gynophores have developed into peanuts.

lished results at the Florida Agricultural Experiment Station indicate that at least calcium is beneficial in the pegging zone and that merely supplying the roots with adequate nutrients will not result in proper nut development.

The purpose of the present investigation was to secure information on the nutrient requirements of the root system as compared to the peg or fruit system.

Methods

The technique of growing peanuts in sand culture with the roots completely isolated from the pegs so that a different nutrient solution can be applied to the roots and to the pegs at the same time has been published (2). The same technique of growing the peanuts was used. Peanuts,

under field conditions in Florida, sometimes develop on the branches, in contact with the soil, small adventitious roots which disappear in dry weather or by harvest time. A string mesh stretched approximately 1 inch above the sand in the pegging area prevented the branches from coming in contact with the fruiting media and the formation of such roots.

Dixie Runner peanuts were planted June 13, 1946. They were inoculated with the proper legume organism. All plants were grown with a complete nutrient solution in the root zone until September 10, 1946, 89

TABLE I

MEAN NUMBER OF GYNOPHORES PER PLANT AND MEAN PERCENT. PER PLANT OF GYNOPHORES PRODUCING FRUIT AS AFFECTED BY WITHHOLDING VARIOUS ELEMENTS FROM THE ROOT OR PEGGING ZONE LAST 51 DAYS OF THE 140 DAYS GROWING PERIOD

TREATMENT[1]		NUMBER GYNOPHORES	GYNOPHORES PRODUCING FRUIT, %	TREATMENT		NUMBER GYNOPHORES	GYNOPHORES PRODUCING FRUIT, %
ROOT ZONE	PEG ZONE			ROOT ZONE	PEG ZONE		
C	C	239	17	O	C	151	18.5
C	O	326	4	–N	C	210	16.5
C	–N	315	16.5	–P	C	230	21
C	–P	315	12	–K	C	273	10.5
C	–K	308	15.5	–Ca	C	215	11.5
C	–Ca	366	1	–Mg	C	191	13.5
C	–Mg	305	11.5	–S	C	339	14.5
C	–S	250	13	–B	C	191	19.5
C	–B	285	12.5	–Cu*	C	228	7.5
C*	–Zn	220	6.5	–Mn	C	427	6
C	–Mn	285	12.5	–Zn	C	255	17.5
C*	–Mo	222	15.5	–Mo	C	206	14.5
C*	–Cu	249	9.5				
L. s. d. (5%)		128.4	5.7			128.4	5.7
L. s. d. (1%)		.....	7.8			.....	7.8

[1] C is complete nutrient solution including the minor elements. Element left out of the nutrient solution after differentiations were begun indicated by negative sign. The O is distilled water.

* Plants were ten days younger, hence not comparable to the others, and the values were not used in the statistical analysis.

days after planting, when differentiation in root and peg treatments were begun. (At that time pegs were developing freely and only distilled water had been applied to the pegging area.) The peanuts were then grown on the different nutrient combinations for 51 days until harvested October 31, 1946, 140 days after planting.

The major elements were applied to the roots at $\frac{1}{4}$ the molar concentration of HOAGLAND and ARNON'S No. 1 nutrient solution (**10**) until July 15, 1946. After this date all major elements were applied at $\frac{1}{2}$ the concentration used by these authors. The minor elements were applied at 1/10 (in parts per million) of the concentration used by Hoagland and Arnon. Some trouble was encountered from the lack of iron so the concentration of that element was changed from time to time, and the foliage

was even sprayed with ferrous salts. The various nutrient treatments are given in table I. They were formulated by modifying or leaving out the elements desired as Hoagland and Arnon did. Distilled water was used throughout the experiment.

Two liters of the proper nutrient solution were applied to the roots of a plant every two days and 1750 ml. of the proper solution twice a week to the pegs of a plant by the method previously described.

The treatments were applied in duplicate and each culture consisted of one plant.

The plants were grown outside on benches and, with the exception of the first few days and the last few days of the period, under one thickness of cheesecloth.

The peanuts were carefully harvested, roots and everything, and both the green and dry weights (at 70° C.) of the various parts of the plant obtained. The fruiting parts (gynophores and peanuts of varying size) were picked off the green plants and divided into three groups as follows: (1) fruit, (2) pegs which had contacted the sand in the fruiting zone but had not appreciably enlarged, and (3) pegs which had not contacted the sand. The first group was considered the fruit yield and consisted of all fruit or gynophores which were enlarging. Some of the smaller of these did not contain seed.

In addition to the main experiment described above, some plants 15 days younger were grown the same way except that the jug containing the roots of a plant was put in a 2 gallon coffee urn liner and around the jug was placed the acid-washed sand in which the pegs could develop. By such an arrangement radioactive elements could be applied either in the rooting or pegging zone as desired. These plants were grown on a complete nutrient solution in both the root and pegging zone until treated with the radioactive elements.[1] Individual plants were selected, and to one, radioactive cobalt was applied in the root area, and to another, in the pegging zone. Different plants were treated in the same way with radioactive phosphorus. A plant was allowed to absorb the element for four days, after which the plant was harvested, by parts, and the amount of the radioactive element in the different parts evaluated by means of a Geiger counter. Cobalt was applied October 10, and phosphorus October 22, 1946. The plants at that time were large and had many pegs.

Results

The roots of the peanuts appeared healthy at harvest time and nodules were present on all of them.

Number of gynophores

The number of gynophores produced per plant and the percentage of

[1] The radioactive studies were conducted through the cooperation of Dr. C. L. Comar, Department of Animal Industry, Florida Agricultural Experiment Station.

those gynophores which develop into fruit are given in table I. In general the differences in peg treatment do not seem to have greatly affected the number of gynophores produced, and deficiencies in the root area seem to have resulted generally in a smaller number of gynophores but there appears to be no particular significance to the differences.

Only a fraction of the total number of gynophores in any case developed into fruit. The lack of calcium in the pegging area and distilled water in the same area resulted in the pegs forming very few fruit.

Total growth

Differences in peg treatment had relatively little effect on total plant growth (table II), but leaving out any one of the major elements in the root zone (with the exception of sulfur), considerably decreased the total yield.

TABLE II

MEAN TOTAL DRY WEIGHT (ROOTS AND EVERYTHING) PER PEANUT PLANT AS AFFECTED BY WITHHOLDING VARIOUS ELEMENTS FROM THE ROOT OR PEGGING ZONE THE LAST 51 DAYS OF THE 140 DAYS GROWING PERIOD

TREATMENT[1]		MEAN WEIGHT, GRAMS	TREATMENT		MEAN WEIGHT, GRAMS
ROOT ZONE	PEG ZONE		ROOT ZONE	PEG ZONE	
C	C	124.4	O	C	59.2
C	O	123.7	–N	C	78.5
C	–N	129.1	–P	C	95.6
C	–P	122.3	–K	C	87.0
C	–K	117.4	–Ca	C	76.8
C	–Ca	122.3	–Mg	C	69.3
C	–Mg	112.1	–S	C	123.1
C	–S	93.1	–B	C	93.7
C	–B	111.1	–Cu*	C	90.0
C*	–Zn	79.1	–Mn	C	134.7
C	–Mn	125.0	–Zn	C	111.8
C*	–Mo	101.7	–Mo	C	86.0
C*	–Cu	87.6			
L. s. d. (5%)		38.2			38.2

[1] C is complete nutrient solution including the minor elements. Element left out of the nutrient solution after differentiations were begun indicated by negative sign. The O is distilled water.

* Plants were ten days younger, hence not comparable to the others, and the values were not used in the statistical analysis.

Peanut yields

The yields of unshelled and shelled peanuts are given in table III. The striking thing about the yields of the peanuts in the hull is the effect of the different treatments in the pegging zone. The results show that distilled water or a nutrient solution minus calcium in this zone resulted in the production of a very small amount of peanuts (table III and figs. 1 and 2), suggesting that calcium is necessary in the pegging zone for good peanut development. No other element left out of the nutrient solution

for the pegging zone resulted in such a failure. However, when sulphur was left out of the solution for this zone there was a statistical significant decrease in yield of peanuts in the hull.

The effect of variation in root treatment on the yield of unshelled peanuts is also given in table III. Leaving calcium, magnesium, or potassium out of the nutrient solution for the root zone gave a statistically significant decrease in yield of unshelled nuts. Leaving any element out

TABLE III

MEAN YIELD OF UNSHELLED AND SHELLED PEANUTS PER PLANT AS AFFECTED BY WITHHOLDING VARIOUS ELEMENTS FROM THE ROOT OR PEGGING ZONE THE LAST 51 DAYS OF THE 140 DAYS GROWING PERIOD

TREATMENT[1]		UNSHELLED NUTS, GRAMS	SHELLED NUTS, GRAMS	TREATMENT		UNSHELLED NUTS, GRAMS	SHELLED NUTS, GRAMS
ROOT ZONE	PEG ZONE			ROOT ZONE	PEG ZONE		
C	C	33.7	23.1	O	C	23.6	15.2
C	O	2.7	0.2	–N	C	26.9	18.6
C	–N	34.5	21.8	–P	C	25.6	14.2
C	–P	24.1	15.0	–K	C	16.8	9.0
C	–K	26.4	14.1	–Ca	C	19.0	13.0
C	–Ca	1.0	0.2	–Mg	C	18.7	13.2
C	–Mg	24.8	15.7	–S	C	31.9	20.0
C	–S	19.1	10.7	–B	C	28.0	18.8
C	–B	27.0	16.1	–Cu*	C	20.8	13.5
C*	–Zn	6.5	2.4	–Mn	C	29.0	18.4
C	–Mn	25.8	15.0	–Zn	C	31.6	19.8
C*	–Mo	27.6	17.4	–Mo	C	27.6	18.0
C*	–Cu	11.1	5.2				
L. s. d. (5%)		14.1	11.4			14.1	11.4
L. s. d. (1%)		19.1	15.4			19.1	15.4

[1] C is complete nutrient solution including the minor elements. Element left out of nutrient solution after differentiations were begun indicated by negative sign. The O is distilled water.

* Plants were ten days younger, hence not comparable to the others, and the values were not used in the statistical analysis.

of the root zone resulted in a lower yield, but only the decreases mentioned above were statistically significant.

No other treatment produced as high a yield of shelled nuts (table III) as a complete nutrient solution in both the peg and root zone, although the absence of only some of the elements gave a statistically significant decrease in the yield of shelled peanuts. The various root and peg treatments had essentially the same effect on the yield of shelled nuts that the same treatments had on the yield of peanuts in the hull, the principal difference being a slight change in the degree of significance.

Top growth

Only two peg treatments had any significant effect on top growth. The highest yield of tops (table IV) were obtained where either distilled

water or the minus calcium treatment was applied to the pegging area. The yields of tops for these treatments are statistically higher than the yield for the complete nutrient solution in both the areas. As has been pointed out, these were the treatments that produced very few peanuts. In other words, the best tops were obtained where nuts failed to develop.

TABLE IV

MEAN DRY WEIGHT OF TOPS PER PLANT AS AFFECTED BY WITHHOLDING VARIOUS ELEMENTS FROM THE ROOT OR PEGGING ZONE THE LAST 51 DAYS OF THE 140 DAYS GROWING PERIOD

TREATMENT[1]		DRY WEIGHT GRAMS	TREATMENT		DRY WEIGHT GRAMS
ROOT ZONE	PEG ZONE		ROOT ZONE	PEG ZONE	
C	C	63.8	O	C	24.7
C	O	82.6	–N	C	27.9
C	–N	68.1	–P	C	46.6
C	–P	64.8	–K	C	46.6
C	–K	66.6	–Ca	C	37.8
C	–Ca	82.7	–Mg	C	34.1
C	–Mg	61.2	–S	C	60.5
C	–S	52.5	–B	C	46.7
C	–B	56.1	–Cu*	C	54.2
C*	–Zn	51.1	–Mn	C	77.0
C	–Mn	69.7	–Zn	C	52.6
C*	–Mo	52.5	–Mo	C	42.8
C*	–Cu	53.7			
L. s. d. (5%)		18.3			18.3
L. s. d. (1%)		24.9			24.9

[1] C is complete nutrient solution including the minor elements. Element left out of nutrient solution after differentiations were begun indicated by negative sign. The O is distilled water.

* Plants were ten days younger, hence not comparable to the others, and the values were not used in the statistical analysis.

On the other hand, some of the root treatments resulting in relatively low nut yields, for example, minus potassium, calcium or magnesium, did not give large tops. Thus there was no very consistant relation between top yield and nut production.

Distilled water, the lack of nitrogen, calcium, magnesium, and molybdenum in the root zone resulted in a significant decrease in top growth. Furthermore, leaving several other elements out of the root zone resulted in poor top growth, but the differences were not sufficient to be statistically significant.

Foliage symptoms

Variations in peg treatments produced no noticeable foliage symptoms. The characteristics of the foliage as affected by a deficiency of the major elements in the root zone were similar to that previously noted[2] but not as

[2] In some unpublished experiments conducted at this station in a similar manner during 1945 by R. W. Bledsoe and the writer, deficient nutrient solutions in the root zone, begun 75 days after germination, resulted in striking foliage deficiency symptoms

pronounced, except that the peculiar leafspot associated with magnesium deficiency (**2**) did not appear this year.

Movement of radioactive phosphorus and cobalt

The preliminary experiments dealing with the intake of radioactive phosphorus and cobalt indicate that the pegs (table V) did absorb a small amount of the phosphorus and cobalt and some of each was translocated to

TABLE V

THE ACCUMULATION OF RADIOACTIVE PHOSPHORUS AND COBALT IN VARIOUS PARTS OF THE PEANUT PLANT AFTER TREATING FOR 4 DAYS THE ROOTING OR PEGGING ZONE WITH THE SAME DOSAGES

	PHOSPHORUS		COBALT	
	PLANT WITH PEG TREATMENT	PLANT WITH ROOT TREATMENT	PLANT WITH PEG TREATMENT	PLANT WITH ROOT TREATMENT
	PERCENT. OF TOTAL DOSE FOUND	PERCENT. OF TOTAL DOSE FOUND	PERCENT. OF TOTAL DOSE FOUND	PERCENT. OF TOTAL DOSE FOUND
ROOTS	0	29.6	0.028	23.9
PEGS AND NUTS	0.04	6.1	0.039	0.83
MAIN BRANCHES	0.008	10.0	0.13	6.3
TIPS OF BRANCHES	0.006	16.2	0.12	6.1
TOTAL PLANT	0.054	61.9	0.317	37.13

other parts of the plant. The roots absorbed more than the pegs, and it should be noted that the pegs and nuts secured far more phosphorus and cobalt through the roots of the plants than did the pegs and nuts directly in contact with the radioactive elements.

Discussion

Peanuts as grown under the conditions of this experiment produced variable yields. This is indicated by the fact that it takes relatively large differences in yields to be statistically significant. More replications would have been desirable.

In these experiments calcium left out of the pegging zone resulted in the formation of few peanuts. Distilled water in the pegging zone gave the same kind of results. The fact that calcium was supplied to the roots all the time it was withheld from the pegs apparently indicates that this

as follows: phosphorus, stunted growth and small bluish green leaves; potassium, stunted growth, dark green leaves and some reddening and dying of the tissue near the tips of the branches; calcium, stunted plant, breaking down of the bud area, and numerous fine brown spots on the leaves, producing a bronze color. A deficiency of almost any of the major elements in the root zone greatly decreased the yield of fruit and fruit failed to develop when only distilled water was applied to the pegging zone.

element cannot be taken up through the roots and translocated to the developing fruit sufficiently to supply the needs in that region. The amount required, the time required, and its function in the pegging area are not known.

The results obtained in these experiments with calcium deficient treatments in the pegging zone apparently are more marked than others have found. WALDRON (**17**) was able to grow two peanuts by putting the gynophores in darkened glass tubes of tap water. Apparently some of the workers in China[3] do not consider nutrients in the pegging zone necessary for fruit development, and SHIBUYA (**12**) of Japan seems to have the same point of view. BRADY, et al., (**4**) appeared to obtain with distilled water treatments about one-fourth of ordinary yields. Why the lack of calcium in the pegging zone had a more pronounced effect in the experiments herein reported than obtained by others is not known, but there might be some varietal difference in response. However, large-seeded peanuts (**7**) are supposed to respond more to lime than small-seeded ones such as the Dixie Runner which was grown in these experiments.

Sulphur was the only element other than calcium when left out of the pegging zone that resulted in a statistically significant decrease in yield of nuts. It, therefore, appears that the sulphate ion in the pegging area is beneficial. In view of the fact that these experiments were conducted in the open where sulphur was brought down by rain, the sulphur requirements of the pegs seem to be more than is in the rain water, and a complete absence of the sulphate ion in the pegging zone might have had a more pronounced effect. These results emphasize the common practice under field conditions of dusting the foliage (**13**) of peanuts with sulphur. The general idea is that the dust controls diseases and for this reason results in an increased yield. In the light of the present experiment this increase in yield could be due in part to the sulphur getting onto the soil and properly supplying the pegs with the sulphate ion, as well as other direct and indirect effects.

BRADY, et al., (**4**) did not obtain a beneficial effect from the sulfate ion in the pegging zone. The reason for this difference in results might be due to varietal differences in response as well as a difference in the amount of sulphur brought down in the rain. Presumably little sulphur is brought down by rains in Florida since the soils in this state frequently respond to the sulfate ion (**1, 9**).

Fertilizers are applied to peanuts under field conditions usually before seeding. Since the peanut is a long growing plant, the nutrient supply probably becomes low during the later part of the growing season. For this reason the different treatments in the root zone were applied the last 51 days of the growing period (140 days) to see what effect late deficien-

[3] Correspondence with Dr. P. S. Tang, Dean, College of Agriculture, National Tsing Hua University, Peiping, China.

cies would have. Several elements when left out of the nutrient solution for the root zone at this late date decreased the yields of peanuts, but the results were not as striking as those referred to in footnote 2 when differentiations in nutrient solutions for the roots were begun at an earlier date. The indications are that various nutrient deficiencies in the rooting zone during the latter part of the growing season will have an effect on the growth of peanuts, but that only the earlier deficiencies are important.

The failure of the plants to develop striking deficiency symptoms probably was due largely to the existence of only slight deficiencies, since the differentiations in nutrient treatments were begun very late.

An accident to some of the plants (indicated in the tables) in the minor element phase of this experiment makes that part of the experiment unsatisfactory. Furthermore, ordinary c.p. chemicals were used without special purification. However, the fact that the lack of molybdenum in the root area seemed to decrease the yield of tops suggests that minor elements might be important in the production of peanuts.

The relative importance of nutrient absorption through the pegs as compared to that through the roots is not well understood. This experiment indicates that calcium is important in the fruiting area and that the sulphate ion is beneficial there. THORNTON and BROADBENT (**15**) found that nitrogen was absorbed by the pegs and that there was a greater absorption of nitrogen by the pegs when the roots were somewhat nitrogen starved. Their results suggest that a deficiency in the root area might increase peg absorption. In the present experiments the plants with minus calcium and minus sulfate peg treatments had liberal amounts of calcium and sulfates in the root area which would further emphasize the importance of these two elements in the pegging zone. The absorption of radioactive phosphorus and cobalt in small amounts by the pegs and the translocation of these elements to other parts of the plant suggest that almost any element might be absorbed in small amounts by the pegs. However, the fact that the pegs obtained far more radioactive phosphorus and cobalt through the roots than when the pegs were directly in the radioactive solution indicates that the roots are more effective in absorbing some elements than the pegs. In view of the limited evidence it would seem that some calcium, and possibly the sulphate ion, is very important in the pegging area, but it is probable that the larger part of most elements in the fruit is obtained through the roots. Possibly an acute deficiency in the root area would increase peg absorption.

Summary

The nutrient requirements of the roots and pegs of Dixie runner peanuts were studied by withholding various elements from the root and pegging zone the last 51 days of the growing season with the following results:

1. Very few fruit developed without calcium in the pegging zone.
2. The sulphate ion in the pegging zone appeared to be beneficial.

3. Leaving several of the major elements out of the root zone the last 51 days of the growth period resulted in lower nut yields but the results are not as striking as previously obtained when differentiation in root treatments were begun earlier in the growing season.

4. The minor element part of the experiment was not conclusive, because of the different ages of some of the plants. However, leaving molybdenum out of the root zone seemed to decrease the yield of tops.

5. Small amounts of radioactive phosphorus and cobalt were absorbed by the pegs and translocated to other parts of the plant, but the pegs received more of these elements when applied to the roots than when the pegs were directly in the radioactive solutions.

6. There was no consistent relation between top growth and nut production.

7. A discussion of the relative importance of root and fruit absorption of nutrients is given. It appears that a balanced supply of nutrients in the root zone as well as calcium and, perhaps, sulphur in the pegging zone are necessary for the best production of nuts.

UNIVERSITY OF FLORIDA
AGRICULTURAL EXPERIMENT STATION
GAINESVILLE, FLORIDA

LITERATURE CITED

1. BLEDSOE, R. W., and BLASER, R. E. The influence of sulfur on the yield and composition of clovers fertilized with different sources of phosphorus. Jour. Amer. Soc. Agron., **39**: 146–152. 1947.
2. BLEDSOE, R. W., HARRIS, HENRY C., and TISDALE, W. B. Leafspot of peanut associated with magnesium deficiency. Plant Physiol., **21**: 237–240. 1946.
3. BRADY, N. C., and COLWELL, W. E. Yield and quality of large-seeded type peanuts as affected by potassium and certain combinations of potassium, magnesium, and calcium. Jour. Amer. Soc. Agron., **37**: 429–442. 1945.
4. BRADY, N. C., REED, J. F., and COLWELL, W. E. The effect of certain mineral elements on peanut fruit filling. Jour. Amer. Soc. Agron., **40**: 155–167. 1948.
5. BURKHART, LELAND, and COLLINS, E. R. Mineral nutrients in peanut plant growth. Soil Sci. Soc. Amer. Proc., **6**: 272–280. 1941.
6. COLWELL, W. E., and BRADY, N. C. The effect of calcium on certain characteristics of peanut fruit. Jour. Amer. Soc. Agron., **37**: 696–708. 1945.
7. COLWELL, W. E., and BRADY, N. C. The effect of calcium on yield and quality of large-seeded type peanuts. Jour. Amer. Soc. Agron., **37**: 413–428. 1945.

8. HARRIS, HENRY C. Some recent findings concerning the nutrition of the peanut. Proc. Asso. Southern Agr. Workers, **44**: 192–193. 1947.
9. HARRIS, HENRY C., BLEDSOE, R. W., and CALHOUN, P. W. Response of cotton to sulfur fertilization. Jour. Amer. Soc. Agron., **37**: 323–329. 1945.
10. HOAGLAND, D. R., and ARNON, D. I. The water-culture method for growing plants without soil. California Agr. Exp. Sta. Cir. 347. 1939.
11. PETTIT, ANNA S. Arachis hypogaea L. Mem. Torrey Bot. Club, **4**: 275–296. 1895.
12. SHIBUYA, T. Morphological and physiological studies on the fructification of peanut. Mem. Fac. Sci. and Agr., Taihoku Imp. Univ., **17**: 1–120. 1935.
13. SMITH, J. LEE. Producing peanuts in Florida. Florida Agr. Ext. Cir. 75. 1944.
14. STOKES, W. E., and HULL, FRED H. Peanut breeding. Jour. Amer. Soc. Agron., **22**: 1004–1019. 1930.
15. THORNTON, G. D., and BROADBENT, F. E. Preliminary greenhouse studies of the influence of nitrogen fertilization of peanuts on nodulation, yield, and gynophore absorption of this element. Jour. Amer. Soc. Agron., **40**: 64–69. 1948.
16. VAN DER VOLK, P. C. Researches concerning geocarpy. Pub. Sur la Physiologie Vegetale, Nimeque, 1914.
17. WALDRON, R. A. The peanut, its history, histology, physiology, and utility. Contr. Bot. Lab. Univ. Pennsylvania **4**: 302–338. 1919.

TETRAZOLIUM CHLORIDE AS A TEST REAGENT FOR FREEZING INJURY OF SEED CORN*

NORAH BENNETT AND W. E. LOOMIS

(WITH ONE FIGURE)

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Rapid methods for estimating viability in seeds have been suggested by a number of workers. Most of these are dependent upon the increased permeability (**6, 10**) or decreased reducing power (**5, 8**) of dead cells or tissues. Staining methods involving the reduction of selenium salts (**4**) or of tetrazolium compounds (**9, 11**) to colored forms by the action of living cells seem to be the most promising quick tests of viability. These tests should be particularly applicable to estimations of freezing injury in seed corn because: (a) corn is easily tested (**11**), (b) immature corn shows a degree of dormancy (**13**) which makes routine laboratory tests of germinability difficult and slow, and (c) speed is always essential to determine whether frozen seed corn is sufficiently viable to justify expensive processing, or whether the crop should be diverted to other uses.

Materials and Methods

Staining tests were limited to the use of 2,3,5-triphenyltetrazolium chloride. The salt was obtained variously from a lot used by PORTER et al. (**11**) and originally obtained from Germany, a lot prepared by the Department of Chemistry, Iowa State College, and several lots supplied by Dr. Nicholas Cheronis of the Synthetical Laboratory, Chicago. Preliminary tests showed that 0.05 per cent. solutions were superior to the 1.0 per cent. used by LAKON (**9**) or the 0.5 per cent. concentration previously used in this laboratory. Corn too dry to cut easily was soaked overnight to 30 or 35 per cent. moisture. Fresh, immature corn was used without soaking. A sample of 50 to 200 kernels was cut carefully with sharp razor blades through the scutellum and embryo axis, and immersed in the tetrazolium solution in petri dishes for two hours at 30° C.

Color readings included records of: (a) intensity of stain, (b) tint, whether purplish, rose or orange red, (c) color texture; viable seeds showed a stippling of deeper staining over the surface of the embryo, (d) the absence, or presence and distribution of unstained areas within the embryo, and (e), in the later readings, the sharpness or disorganization in cellular structure of the stained tissue. In the directions given by LAKON (**9**) and PORTER et al. (**11**) corn was considered viable if the embryo stained pink

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under these conditions. Lakon recognized necrotic (unstaining) areas in old or injured seeds, and attempted to establish the amount of necrosis indicating abnormal or nongerminable seeds.

Preliminary tests were run on several samples of old corn of varying germinability and later on mature grain soaked to the desired moisture percentages and frozen before testing. The main series of experiments was run with corn of varying degrees of maturity, ears of which were given varying freezing treatments in a low temperature chamber. Some of these lots were tested immediately after freezing and some after various holding or drying and resoaking treatments.

Tests of Old Seeds

Three lots of old corn from different sources were used for preliminary tests of the tetrazolium method. Twelve samples of 6-year old corn were

TABLE I

GERMINATION AND STAINING PERCENTAGES OF CORN SEED STORED SIX YEARS IN THE LABORATORY

CLASSIFICATION	SAMPLE 1	SAMPLE 2	SAMPLE 3
Germination in sand			
Normal	36	80	48
Abnormal but not entirely dead	14	2	24
Dead	50	18	28
Tetrazolium tests			
Strong staining	20	74	6
Pale staining	0	6	0
Scutellum weakly stained	0	0	8
Small unstained area at base of scutellum	0	18	64
Few scattered unstained areas in scutellum	22	0	0
Total considered normal	42	98	78
Larger unstained area at base of scutellum	0	0	20
Several scattered unstained areas in scutellum	56	0	0
Shoot apex colorless	0	2	0
Entire embryo colorless	2	0	2

obtained from Dr. E. W. Lindstrom of the Department of Genetics, 16 samples one to four years old were supplied by Northrup, King and Co., and six samples of unknown history were obtained from the College Seed Laboratory.

Of the 12 samples obtained from Dr. Lindstrom, only three germinated below 98 per cent. in sand. One of these, designated sample 1, showed scutellar injury in the tetrazolium test, indicated by scattered areas which failed to stain. The other two showed localization of the unstained areas at the base of the scutellum. In all cases the necrotic area was less than the one-third of total area used by LAKON (**9**) as a measure of loss of viability. The data of table I show the sand test germination of these three lots and their tetrazolium staining reactions. It appeared that the estima-

tion with tetrazolium of seeds considered normal was approximately correct only for sample 1. Germination test for sample 2 indicated that in this lot those seeds with small unstained areas at the base of the scutellum were dead, but in the third sample similarly stained seeds were viable and more than half of them were capable of producing normal seedlings.

The samples obtained from the Northrup, King and Co. were high in germinability, with only one sample going below 90 per cent. This sample was estimated at 100 per cent. by the tetrazolium test. Other estimates agreed within the usual errors of testing. Seeds which germinated abnormally apparently were not separated by the tetrazolium test.

Similar results were obtained with the third set of samples. A reasonably good estimate of normal seedlings was possible, but seeds producing abnormal seedlings were not accurately separated from dead, or sometimes from normal.

Tests of Frozen Seeds

The first tests on frozen seed were with four samples supplied by Mr. M. M. Aboul-Ela from his experiments on chemical changes in frozen corn

TABLE II

GERMINATION AND STAINING PERCENTAGES OF FOUR LOTS OF CORN FROZEN IN THE FALL OF 1946 AND TESTED IN MAY, 1947. (50 TO 100 SEED SAMPLES)

Sample	Germination in sand				Tetrazolium staining			
	Moisture % when frozen	Normal	Abnormal	Dead	Complete stain	Pale stain	Partial stain	No stain
1	62.5	92	1	7	86	0	14	0
2	41.1	97	1	2	100	0	0	0
3	56.6	3	0	97	4	6	2	88
4	39.9	10	0	90	10	4	4	82

(**1**). All samples were frozen as snapped ears for six hours at 20° F. and then hung back on the stalks to dry in the field. Samples 1 and 2 were from an early planting which dried rapidly under favorable conditions. Samples 3 and 4 were from later planted corn and were subjected to several weeks of weathering during the drying period. These seeds had either been killed by the freezing treatment or had recovered (**12**) and the time lapse of about six months between the freezing treatment and testing was sufficient for the establishment of clear-cut staining differences (Table II). The variations between complete stain by tetrazolium and normal germination in sand can be ascribed to sample variation.

A second group of samples of frozen seed was obtained from Mr. Elmer C. Rossman. These samples were given varying freezing treatments while still on the ear. After freezing they were allowed to thaw for six to eight hours before being placed in corn-seed drier to dry at approximately 95° F. In the tetrazolium solution all seeds showed staining of the scutellar region.

TABLE III

PERCENTAGE GERMINATION AND STAINING OF SIX LOTS OF FROZEN CORN. GERMINATION BASED ON 35–129 SEEDS; TETRAZOLIUM ON 100

	Germination tests					Tetrazolium tests			
Sample	Normal	No primary root	Total germinable	Abnormal	Dead	Complete staining	Plumule unstained	Radicle unstained	Embryo axis unstained
1	55	6	61	36	3	87	13	0	0
2	5	0	5	15	80	16	0	0	84
3	86	0	86	14	0	85	0	15	0
4	63	0	63	37	0	71	0	29	0
5	45	4	49	49	2	65	0	35	0
6	8	1	9	2	89	8	0	0	92

Those counted non-germinable showed no staining either in the plumule or in the radicle or in both of these areas. Seedlings germinating without the primary root but with well developed seminal roots were included in the total of germinable seeds. Table III shows the comparison of germination and staining tests. The differences in the germination of these samples is credited to differences in the moisture content at the time of freezing. In all but two comparisons the tetrazolium estimation of viable seeds tended to be slightly higher than the germination test, regardless of the type of injury evidenced by lack of staining in certain regions. Partial loss of vitality as a result of freezing seemed to result in an overestimation of germinability when samples were tested by the tetrazolium method.

The foregoing tests indicated that more information on the effect of tetrazolium on frozen seeds was desirable. Because other frozen samples were not available at this time, normally matured and cured seed which had been soaked in water and then frozen was used. Seed from Northrup, King sample 3171A8 was soaked in water at 15° C. to varying moisture contents. The seeds were then drained, blotted to remove excess water and frozen for 24 hours at 20 and at 0° F. After thawing the samples were cut and placed in the tetrazolium solution, with duplicate samples planted in sand. In each case two lots of 100 seeds were used. Table IV contains the data from the two methods.

It was apparent that staining alone was not a criterion of viability in corn seed which had recently been subjected to freezing injury, as there were great differences in the estimation of viability by the two methods in the more severely frozen seeds of higher moisture content. It was discovered that the position of these injured seeds in the tetrazolium solution had a bearing on the intensity of stain produced. However, injured seeds, even though deeply stained, were different from viable seeds. It was noted that: (a) in dead seeds there was much more prominent contrast of color intensity between embryo axis and scutellum, the former being darker; (b) the embryo axis appeared to be blurred without the outlines of the foliage leaves of the plumule being visible; (c) the scutellum had lost the more or less granular appearance characteristic of the living tissues. Retesting with tetrazolium of those samples which showed divergence from the germination test, and using these factors as the basis of separation, gave results closely approximating the sand germination test results.

Tests of Fresh Seeds

The main objective of this study was to evaluate the use of tetrazolium chloride for rapid tests of freshly frozen, immature seed corn. Immature seed required up to eight weeks to germinate in sand, giving a lower average value than dried seed even after this time. On the other hand, Rossman (**12**) found that quick drying, such as would be required for rapid conventional tests of frozen corn, may further reduce viability. We attempted, therefore, to correlate a quick tetrazolium test with sand germin-

TABLE IV

PERCENTAGE GERMINATION AND TETRAZOLIUM STAINING OF CORN FROZEN AT INCREASING MOISTURE CONTENT. (2 LOTS OF 100 SEEDS EACH)

SERIES	MOISTURE (WET WT. BASIS)	FREEZING TEMPERATURE, °F.	GERMINATION IN SAND			TETRAZOLIUM STAINING			REPEATED TETRAZOLIUM
			NORM.	ABN.	DEAD	FULLY COLORED	WHITE SPOT*	COLORLESS	NORMAL
1	18.2	20	99	1	0	87	13	0	
		0	98	2	0	88	12	0	
2	19.6	20	95	4	1	88	10	2	
		0	98	1	1	87	12	1	
3	23.1	20	97	2	1	93	6	1	
		0	68	3	29	59	10	31	
4	25.5	20	98	2	0	87	11	2	
		0	62	4	34	63	26	11	
5	28.1	20	92	0	8	90	0	10	89
		0	11	6	83	32	48	20	10
6	32.0	20	76	8	16	77	5	18	82
		0	2	0	98	74	6	20	1
7	32.9	20	55	15	30	72	19	9	63
		0	0	0	100	47	2	51	0
8	36.1	20	7	0	93	60	26	14	7
		0	0	0	100	.....	.....	.....	0

* In scutellum.

ation after rapid and after slow drying, sometimes with seed from the same ear and sometimes on a composite sample when seed quantities were too small for ear by ear comparisons.

Material for these tests was supplied and freezing treatments made by Mr. M. M. Aboul-Ela. Ear remnants for the sand tests were dried at 95° F., either at once or after 3 or 10 days of slow drying in the field. Our tests did not show any consistent differences in the germination of corn dried rapidly immediately after freezing or ten days after freezing, and these treatments are combined in the data.

Three collections were made, the moisture contents of which were approximately 60, 50 and 40 per cent. Ears were selected from the field at random and divided into three groups for treatment. One group of ears was

TABLE V

COMPARISON OF MEAN PERCENTAGE VIABILITY ESTIMATES OF TETRAZOLIUM TESTS OF UNDRIED EARS, WITH GERMINATION TESTS OF THE SAME EARS AFTER RAPID DRYING.

Series	Moisture at freezing	Freezing treatment	Tetrazolium		Germination		Mean diff.	Stand. dev.	"t"	Freezing temperature
			No. of tests	Mean	No. of tests	Mean				
1	58.3	unfrozen	22	99.7	13	95.2	− 4.5	.86	5.23*	20° F.
		8 hours	22	77.1	18	64.5	−12.6	4.98	2.53*	
		16 hours	22	31.8	18	18.4	−13.4	5.50	2.44*	
2	50.3	unfrozen	30	99.8	13	97.2	− 2.6	.77	3.39*	20° F.
		8 hours	30	61.1	10	54.0	− 7.1	6.51	1.09	
		16 hours	30	32.7	9	19.9	−12.8	5.30	2.42	
3	39.4	unfrozen	31	99.7	9	98.9	− .8	1.98	.40	10° F.
		8 hours	25	17.4	10	22.2	− 4.8	4.67	1.02	
		16 hours	25	1.6	9	3.8	− 2.2	4.26	.52	

* Significant at 5% level.

left unfrozen, a second was frozen for eight hours and a third for 16 hours at temperatures of 20 or 10° F. Ears from each treatment were then subdivided into three lots. The first of these was tested immediately, the other two were returned to the field to be dried on racks. One of these lots was tested after three days, the other after ten days in the field. Duplicate samples from each ear were tested by the tetrazolium method and by the germination test. Seed for these tests was removed from the ears in longitudinal rows. The ear remnants were then dried in the corn-seed drier and retested by both methods. Table V presents the summarized data of tetrazolium tests of undried and sand tests of dried samples.

All fresh seeds stained completely when placed in the tetrazolium solution, regardless of freezing treatment. While there was no noticeable difference in the color intensity produced in immature samples, there were differences in the shade of color. The color of the embryo axis was purplish in frozen seeds. Seeds tested three days after freezing could be dis-

tinguished by a white film which masked the staining. This film became more pronounced as the time between freezing and immersion of the cut seeds in the tetrazolium solution was increased. Dead seed tested after ten days exposure in the field was colorless or almost colorless. Color differences were more noticeable in the staining of the more mature fresh seeds. Those assumed to be viable were generally paler and more orange in color. The structure of the viable seed was clearly visible, whereas in frozen seed the outline of the germ was blurred and its internal structure

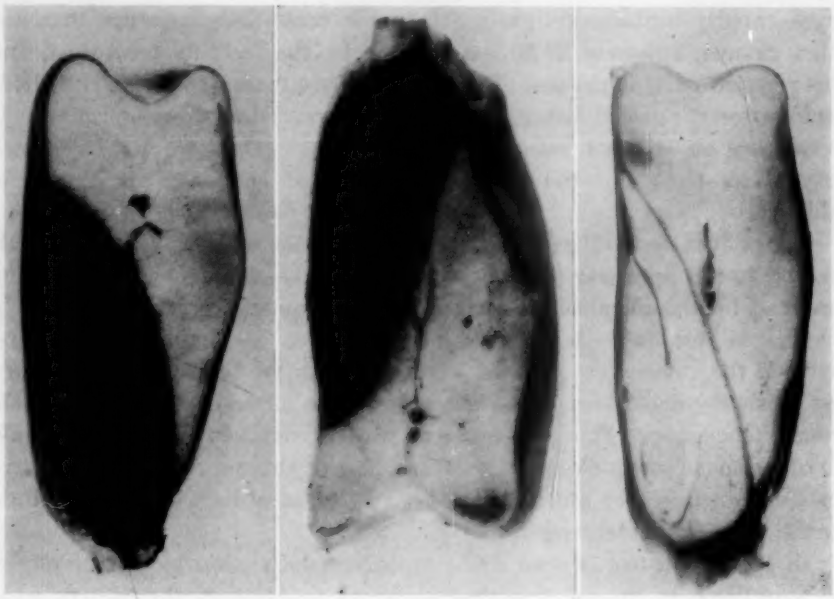

FIG. 1. Staining of maize grain in tetrazolium solution. Left, normal seed; center, freshly frozen seed showing heavy, blurred staining; right, frozen seed 1 week later showing typical Lakon stain for dead grain.

could not be distinguished clearly. Some idea of the differences observed may be obtained from figure 1.

Formation of color by the reduction of the tetrazolium solution was not a criterion of viability. The color produced by reduction of the compound accentuated the differences in viable and non-viable seeds, however, so that separation was possible. Our readings with tetrazolium tended to be five or ten per cent. higher than the actual vitality indicated by the germination test. As stated above, we had no evidence that this difference was due to the drying treatment.

Effect of Rate of Drying

Tests were made to determine whether there was an optimum period after freezing for testing with tetrazolium, at which time the separation of

live and dead seed could be made with the greatest accuracy; also to determine whether the rate of drying after freezing would affect this optimum.

Twenty ears of immature corn containing from 40 to 45 per cent. moisture were used in these tests. Ten ears were frozen at 20° F. for eight hours and ten for 16 hours. The first tetrazolium test was made about one hour after removal from the freezing chamber. At the completion of this preliminary test, five ears from each freezing treatment were left to dry at room temperature, while the remaining five ears from each group were dried rapidly in a hot air current. Ears dried slowly were retested after 24, 48 and 96 hours. The group of ears frozen for eight hours and dried rapidly contained slightly (three per cent.) less moisture than the other groups, appeared to be less affected by the freezing treatment, and was retested only at the end of 24 hours. Ears frozen 16 hours and dried rapidly were retested after 24 and 48 hours. Moisture determinations were made on each ear before and after drying. Data from the repeated tetrazolium tests and the germination tests of each ear are shown in table VI.

In the first tests all seeds stained. Separation of dead seed was made possible by the appearance of a whitish film on the cut surface of the frozen seed and by the purplish color and blurred appearance of the germ. In the second test of seeds dried slowly, the seeds all stained. The white layer on the cut surface of dead seeds seemed to be thicker. In the third test of slowly dried seed, the white appearance of dead seed was very obvious. The embryo axis of some seeds was entirely white while the region surrounding it was pinkish. In the last test of this series some seeds were completely white. In others the embryo axis was white with the scutellum partially or completely pink.

In the second test of seed dried rapidly, dead seeds which were placed cut side down in the solution showed deep coloration of the embryo axis. Frozen seeds not in this position were pinkish-white. In the third test of rapidly dried seeds, dead seeds were very pale. In some, the embryo axes were white, while the scutellar region was colored. No increase in the accuracy of the tetrazolium tests, as we read them, resulted from a delay after freezing. The means of the tests for each time interval were very close. Except in the second group, the tetrazolium readings were significantly higher than those obtained from the sand tests.

There appeared to be considerable variation in tetrazolium tests of seed from the same ear. To determine whether this variation was due to misinterpretation of staining or was caused by differences in germinability, all seeds from three frozen ears were planted in sand. Each ear was divided transversely into three sections. Each section was divided longitudinally into four parts for planting. The lowest third of the ear gave the highest germination and the upper third the lowest in each comparison, with differences of more than 30 per cent. on two of the three ears.

TABLE VI

EFFECT OF RATE AND AMOUNT OF DRYING OF FROZEN CORN ON THE TETRAZOLIUM READINGS

TREATMENT	MOISTURE % AS FROZEN		GERMINATION	STAINING				MOISTURE AT END
				1 HR.	24 HRS.	48 HRS.	96 HRS.	
FROZEN 8 HOURS, FAST DRY.	40.7		95	100	95			20.8
			84	98	97			
			96	92	99			
			95	96	97			
			87	87	86			
		Mean	91.4	94.6	94.8			
FROZEN 16 HOURS, FAST DRY.	43.6		48	48	52	49		17.5
			66	51	59	52		
			81	71	73	79		
			62	53	58	57		
			57	50	50	46		
		Mean	62.8	54.6	58.4	56.6		
FROZEN 8 HOURS, SLOW DRY.	45.7		58	68	62	57	70	36.2
			71	74	75	75	76	
			83	70	80	83	72	
			74	81	82	82	75	
			52	85	83	74	74	
		Mean	67.6	75.6	76.4	74.2	73.4	
FROZEN 16 HOURS, SLOW DRY.	42.2		40	66	71	64	54	34.2
			52	48	51	53	51	
			45	52	52	50	52	
			39	41	49	61	55	
			69	81	73	74	86	
		Mean	49.0	57.6	59.2	60.4	59.2	

Effect of pH

The preparation of 2,3,5-triphenyltetrazolium chloride involves purification of the salt from hydrochloric acid, and the acidity of the resulting product varies with the method of preparation. To determine whether such variations in acidity might have an effect on the staining qualities of the compound, buffer solutions were made with mixtures of citric acid and disodium phosphate (McIlvaine's standard). The pH determinations were made on a Leeds and Northrup glass electrode pH meter. The sample of corn used was one which had been frozen while immature. It

had a germination of 70 per cent. and an additional 12 per cent. of seeds which developed abnormally.

In the first test the seeds were soaked overnight, then cut and placed in the buffer solutions with 0.5 per cent. tetrazolium chloride. Dishes containing the samples were kept at 30° C. for two and one half hours, and at the end of that time the staining in the different solutions was compared. It was found that viable seeds stained intensely from pH 8.0 to 6.8. From 6.6 to 6.0 the staining became progressively paler although it was sufficient to distinguish viable from dead seeds. Below 5.0 the staining was too pale for satisfactory readings. All viable seed appeared to stain comparably at each pH level, with no differentiation of seeds which might produce abnormal seedlings.

In a second experiment, uncut seeds were soaked overnight in the buffer solutions, after which they were cut and treated in the usual manner. At the conclusion of the tests it was found that all viable seeds stained adequately, although staining was paler in seeds presoaked in buffers with pH less than 5.0. Again abnormal seeds could not be distinguished by their staining differences.

Determinations of pH were made on solutions of different lots of tetrazolium chloride. The pH range was from 9.5 to 3.3. Solutions of all these samples showed no differences in the staining of viable seeds.

When the pH of the buffered tetrazolium solutions was below 5.0 the efficiency of the reducing agent was decreased so that only a pale stain resulted. In unbuffered solutions, however, the pH of the solution was unimportant. Staining was affected only slightly by soaking the seed in the buffered solutions before testing. It is probable that the cells of the tissue have a strong buffering action. It would appear, therefore, that variations in the pH of the tetrazolium solutions normally used in testing have little effect on staining.

Discussion

The tetrazolium test is a test of reducing enzymes or substances within the seed rather than of viability. One of the authors (**2**) has been unable to show a satisfactory correlation between peroxidase activity, used as an index of enzyme action, and tetrazolium reduction in frozen corn. Peroxidase remained constant while staining first increased and then decreased to near zero. It seems probable, therefore, that labile reducing substances normally maintained, or produced in the viable seed during soaking, are the basis of the Lakon test. The first increase of staining characteristic of freshly frozen seed is probably due to increased permeability and more rapid absorption of stain. The decreased tendency to stain with tetrazolium developed most rapidly when the frozen seeds were dried slowly at room temperatures, but it was usually several days before the presence or absence of stain in the embryo could be used as an index of viability. On the other hand it was not difficult to measure other differences in the

staining and appearance of frozen embryos which made it possible to estimate their viability immediately after freezing. While the criteria used here, tint, mottling, texture, etc., are difficult to describe, they can be read by an experienced observer. (Cf. fig. 1.)

Since increased permeability is a characteristic (3) and immediate response of cells to freezing injury, tests of permeability with various dyes suggest themselves for quick measurements of freezing injury to corn. The tetrazolium test also is affected by permeability, however, as indicated above, and so is useful in early as well as in later tests of injury.

The uniformly high estimates of viability obtained with the tetrazolium seem to be largely the result of partial injury in seeds which are actually "viable" but not "germinable" in sand in the sense of being capable of producing a normal seedling.

Since this manuscript was prepared Goodsell has published a study of the staining of frozen maize with tetrazolium (7). His results agree with ours in showing that the Lakon test of pink staining is not applicable to freshly frozen, immature corn. Goodsell's recommended method is to dry the frozen corn rapidly and resoak before staining. He notes that even this procedure was not successful with a sample frozen at 51 per cent. moisture. By changing the basis of reading the tests, we have been able to estimate freezing injury immediately in corn frozen with as much as 60 per cent. moisture.

Summary

Freezing injury of seed corn could be estimated with fair accuracy from the development of embryo staining in a 0.05 per cent. solution of 2,3,5-triphenyltetrazolium chloride, provided the germination was moderately high and the corn had been stored for some time after freezing. The tetrazolium readings were commonly higher than the germination tests in sand, and did not give satisfactory estimates of percentages of abnormal seedlings.

When tetrazolium was used on freshly frozen, immature corn of 30 to 60 per cent. moisture content, staining of dead kernels tended to be more intense rather than spotted or absent as expected from tests of old seeds or those killed in hot water. This intensity we ascribe to increased permeability of the injured cells to the dye, which is then reduced to the colored form by substances present in the tissues before freezing. The dead kernels slowly lose the ability to reduce tetrazolium, and after several days or weeks can be tested by Lakon's method.

With experience, however, it is possible to estimate the injured seeds immediately after freezing, using tint, texture, structural appearance and other characteristics of the injured tissue instead of the presence or absence of a pink stain. The accuracy of such early estimates is not less than that obtained later. All tetrazolium tests tend to give a high estimate of germinability, particularly of badly injured seed, and the method

is not recommended for critical testing. It provides a means, however, for estimating the severity of frost damage to corn within two hours instead of one or two weeks.

IOWA STATE COLLEGE
AMES, IOWA

LITERATURE CITED

1. ABOUL-ELA, M. M. Physiological changes in maturing maize. Unpublished M. S. Thesis. Iowa State College Library. Ames, Iowa. 1947.
2. BENNETT, NORAH. Tetrazolium chloride as a test reagent for freezing injury of corn seed. Unpublished M. S. Thesis. Iowa State College Library. Ames, Iowa. 1948.
3. DEXTER, S. T., TOTTINGHAM, W. E., and GRABER, L. F. Investigations of the hardiness of plants by measurement of electrical conductivity. Plant Physiol. **7**: 63–78. 1932.
4. EIDMANN, F. E. Eine neue biochemische Methode zur Erkennung des Aussaatwertes von Samen. Int. Seed Test. Assn. Proc. **10**: 203–211. 1938.
5. GADD, IVAR. Colouring of pea seeds by means of malachite green. Int. Seed Test. Assn. Proc. **13**: 5–76. 1941–1943.
6. ————————, and KJAER, ARNE. Über die Verwendbarkeit der Selen- und Indigokarminmethoden bei der Prüfung von Frost- und Fusariumgeschädigtem Getreide. Int. Seed Test. Assn. Proc. **12**: 140–149. 1940.
7. GOODSELL, S. F. Triphenyltetrazolium chloride for viability determination of frozen seed corn. Jour. Amer. Soc. Agron. **40**: 432–442. 1948.
8. HASEGAWA, KOZO. On the determination of vitality in seed by reagents. Int. Seed Test. Assn. Proc. **7**: 148–153. 1935.
9. LAKON, GEORG. Topographischer Nachweis der Keimfähigkeit von Mais durch Tetrazoliumsalze. Ber. Deutsch. Bot. Ges. **60**: 434–444. 1942.
10. NELJUBOV, N. Vitalfarbung von Samen. Schriften für Samenkunde. Zapiski po Semenovedeniju. 1925. (Original not seen; cited by Gadd and Kjaer, Int. Seed Test. Assn. Proc. **12**: 140–147. 1940.)
11. PORTER, R. H., DURRELL, MARY, and ROMM, H. J. The use of 2,3,5-triphenyltetrazolium chloride as a measure of seed germinability. Plant Physiol. **22**: 149–159. 1947.
12. ROSSMAN, E. C. Viability and vigor of inbred and hybrid maize seed subjected to freezing temperatures. Unpublished Ph.D. Thesis. Iowa State College Library. Ames, Iowa. 1948.
13. SPRAGUE, G. F. The relation of moisture content and time of harvest to germination of immature corn. Jour. Amer. Soc. Agron. **28**: 472–478. 1936.

BRIEF PAPERS

SOME ASPECTS OF THE STATE OF CHLOROPHYLL IN CHLOROPLASTS

W. W. Lepeschkin

(WITH TWO FIGURES)

Received April 3, 1948

As is known, chlorophyll can be removed from chloroplasts by alcohol independently whether the leaves containing them are fresh or dry. If the alcoholic solution of chlorophyll containing a small quantity of water is shaken with benzene, all chlorophyll is transferred to benzene showing that it is much more soluble in benzene than in alcohol. In spite of this fact chlorophyll cannot be extracted from leaves by benzene. Chlorophyll cannot be extracted from fresh leaves by means of benzene because this reagent is immiscible with water. It is more difficult to explain the inability to extract chlorophyll from dry leaves with benzene. The data of this paper discuss this point. The impossibility of extracting chlorophyll from dry leaves by benzene can be explained by assuming that it is chemically combined with some substance in the chloroplasts (**1, 2**), but at the same time it is evident that such a compound is very unstable and is destroyed by alcohol.

Compounds of proteins and lipids have been produced artificially (**3, 4**). It is possible to introduce chlorophyll into such a complex and to compare its properties with those of chloroplasts. Since chloroplasts contain considerable amount of water, are liquid or gelatinous and their substance is immiscible with water solutions, their physical state can be compared with that of fluoids (or "coacervates"). The lipoproteinic complex containing chlorophyll should be also in the same physical state. Lipoproteins in the state of fluoids were recently produced (**5**).

A complex of albumose and lecithin, containing chlorophyll, in the state of fluids, was obtained in the following manner: a 15 per cent. solution of peptone Witte (Merck) was boiled and filtered. The filtrate contained only albumose. Its pH was set at 8.5 by addition of KOH. To 1.6 cc. of this filtrate, 0.4 cc. of a four per cent. alcoholic solution of lecithin was added drop by drop and two cc. of a saturated solution of ammonium sulfate was added to the mixture. Then 3.5 cc. of a concentrated alcoholic extract of leaves of Gramineae was added and the liquid poured into a 10-cc. glass cylinder. After some hours, three layers became distinct in the liquid. The upper layer had a yellowish tinge, the middle one was green, and the lower layer was slightly yellowish. The microscopical investigation of the green layer showed that it consisted of large droplets of a con-

centrated solution of ammonium sulfate on the surface of which green droplets of the albumose-lecithin fluoid containing chlorophyll were attached. These droplets congealed soon but after adding a small quantity of water (0.5 cc.) to the liquid in the cylinder they became liquid again, and in few days many of them had flowed together forming large green

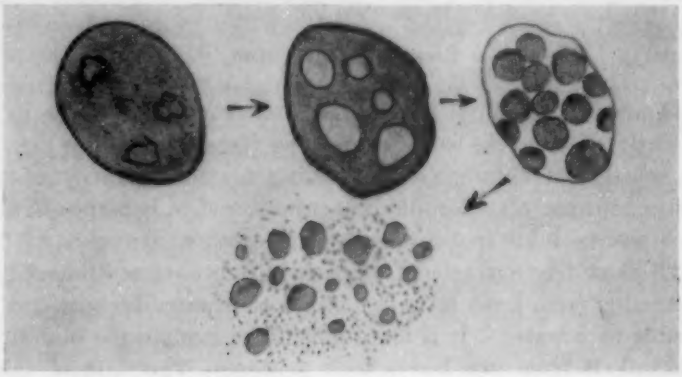

Fig. 1. Effect of water on fluoid drops of lecitho-albumose containing chlorophyll.

drops. Their viscosity was rather great but they had a regular spherical surface. Some of them contained vacuoles. The aggregate state and size of the green drops could be changed. Upon addition of ammonium sulfate to the mixture they congealed but addition of water liquefied them. Addition of water to a microscopic preparation containing the fluoid caused the droplets to absorb water and vacuoles appeared in their interior. A new addition of water produced the separation of a colorless substance from the green drops. The vacuoles disappeared and the green substance formed

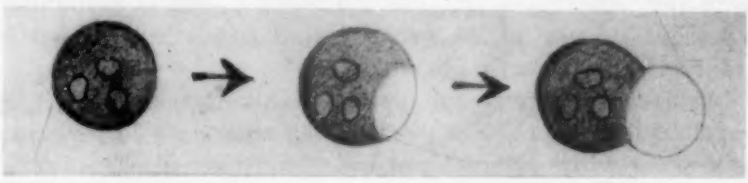

Fig. 2. Effect of water on chloroplasts of Bryopsis.

droplets while the colorless substance surrounded them (fig. 1). An analogous response is obtainable with the chloroplasts of Bryopsis and the effects of distilled water upon chloroplasts of Bryopsis in sea water ~~(fig. 2)~~. *is shown in figure 2* As is known, ether added to a preparation containing chloroplasts dissolves in their lipids forming green droplets (**6, 7, 8**). When ether is added to the preparation containing viscous droplets of a complex albumose-lecithin-chlorophyll, it dissolves in such droplets and decreases their viscosity. Simultaneously small vacuoles appear in them and finally green droplets.

If alcohol is added to the microsopic preparation of droplets of albumose-lecithin complex containing chlorophyll, the droplets shrink and congeal but remain green and only a part of chlorophyll dissolves in the surrounding liquid. Chlorophyll cannot be removed from chloroplasts by means of diluted alcohol. However, chloroplasts as well as droplets of the albumose-lecithin complex containing chlorophyll lose their chlorophyll if treated with strong alcohol. If the complex of albumose-lecithin-chlorophyll is dried, benzene cannot extract chlorophyll from it though alcohol extracts it easily.

An alcoholic solution of Sudan II added to the preparation of the complex stains them brown. Water in this case also causes a separation of colorless and colored substances but the resulting small droplets are brown. As is known, an alcoholic solution of Sudan II also stains chloroplasts brown.

The complex consisting of albumose, lecithin and chlorophyll thus seems similar to chloroplasts in its aggregate state and in reaction to water, ether, alcohol and benzene. We may conclude that chlorophyll in chloroplasts probably takes part in the formation of a lipoproteinic complex.

NAVAL MEDICAL RESEARCH INSTITUTE
NATIONAL NAVAL MEDICAL CENTER
BETHESDA, MARYLAND

LITERATURE CITED

1. LUBIMENKO, W. Condition de chlorophyll aux plastide. C. R. Acad. Sci. Paris. **173**: 365–367. 1921.
2. OSBORNE, T. B. The chemistry of the cell. Bull. Soc. Hyg. **18**: 1–8. 1930.
3. PRZYLECKI, ST., and HOFER, E. Untersuchungen über kunstliche "Lipoproteide." Bioch. Zeitschr. **288**: 303–309. 1936.
4. CHARGRAFF, E., and MORRIS SIFF. The compounds between phosphotides and basic proteins. J. Biol. Chem. **131**: 25–34. 1940.
5. LEPESCHKIN, W. W. Über flüssig-flüssige Entmischung einiger Lipoproteide. Table of microphotographs. Kolloid Zeitschr. **107**: 131–134. 1944.
6. LEPESCHKIN, W. W. Chemische Zusammensetzung der lebenden Materie. Kolloidchemie des Protoplasmas II. ed. Dresden (Steinkopf) 1938.
7. WEBER, F. Myelinfiguren und Sphärolithe aus Spirogyra-Chloroplasten. Protoplasma **19**: 455–460. 1933.
8. MENKE, W. Untersuchungen über Chloroplasten. Protoplasma **21**: 279–298. 1934.

THE ACTION OF GROWTH INHIBITORS ON CARBOHYDRATE METABOLISM IN THE PEA[1]

G. S. Christiansen, L. J. Kunz, W. D. Bonner, Jr. and K. V. Thimann

Received June 11, 1948

Sufficient evidence has accumulated in the past few years to make it certain that growth by cell enlargement is inseparably connected with certain aspects of metabolism. In the Avena coleoptile, growth of isolated sections in simple solutions containing auxin is strongly aerobic and is inhibited by inhibitors of oxidizing and dehydrogenating enzymes. In particular the inhibiting action of iodoacetate, arsenite and fluoride on growth in presence of auxin [Thiamann and Bonner (**4**) and unpublished data] suggests a relationship with carbohydrate metabolism, since these substances are all known to inhibit enzymes acting on the breakdown of carbohydrates. This paper is a preliminary report on a series of experiments designed to elucidate the relation between these three inhibitors and the metabolism of carbohydrates.

Since Avena coleoptile sections only grow well in the presence of sucrose, pea stems were used instead. Sections 20 mm. long were cut from the apical portion of 7-day-old etiolated Alaska pea plants and floated on the test solutions in darkness at 25° C. for 24 hours. They were then weighed, measured, and at once extracted with boiling 80% alcohol. Sugars were determined by the Shaffer-Hartman-Somogyi procedure (**3**).

In general such 20 mm. sections grow about four mm., or 20%, in water alone, and about 10 mm., or 50%, in indoleacetic acid (1 mg./l.). The growth is thus small but is very reproducible and has been shown to take place almost wholly in the first 12 hours. The reducing sugar content of the stem sections averaged 1.12% of the fresh weight and after 24 hours' growth this fell to about three-fourths of the initial value. From a series of concentration curves for each inhibitor, which will be published in detail later, points corresponding to 50% inhibition were selected. The data for growth and reducing sugar content at this and at a higher concentration for each inhibitor are presented in table I.

It will be seen first that in general the increase in fresh weight parallels the increase in length, with minor variations. Secondly, whether the sections are held in water or in auxin the disappearance of reducing sugar is the same. The data for the water and auxin controls are each the mean of 15 experiments. The concentrations of inhibitors needed for 50%

[1] Part of this work was made possible through a grant from the Committee on Growth of the National Research Council, acting for the American Cancer Society, to whom we are indebted.

growth inhibition vary considerably, arsenite being the most active and fluoride the least. However, in spite of the differences of absolute concentrations required, the effects on the reducing sugar content are qualitatively the same, namely, that inhibition of growth is accompanied by a further fall in the reducing sugar. In other words, more reducing sugar appears to be consumed in presence of the inhibitor, in spite of the fact that it is

TABLE I

GROWTH AND REDUCING SUGAR OF 20 MM. SECTIONS OF ETIOLATED PEA STEMS AFTER 24 HRS. IN SOLUTION

	GROWTH		REDUCING SUGAR	PER CENT DECREASE	
	AS PER CENT ELONGATION	AS PER CENT INCREASE IN FRESH WEIGHT	AS PER CENT OF FRESH WEIGHT	OF GROWTH IN LENGTH	OF REDUCING SUGAR
Initial	.....	.....	1.12	.....	.....
In water	20.0	22.2	0.83	.....	.....
In auxin (1 mg./l.)	50.9	58.8	0.86	0	0
In auxin plus:					
Iodoacetate					
6×10^{-4} M	25.6	22.6	0.64	50	26
1×10^{-3} M	18.2	20.9	0.54	64	37
Arsenite					
1×10^{-4} M	26.5	24.0	0.71	48	17
1×10^{-3} M	0.0	5.3	0.51	100	41
Fluoride					
5×10^{-3} M	25.3	33.7	0.48	50	44
1×10^{-2} M	17.8	20.6	0.42	65	51

not being used in growth. In the case of fluoride, the fall in reducing sugar for a given decrease in growth is greater than that for the other inhibitors.

An attempt was made to determine whether the reducing sugar had entered into other combinations. Analyses for sucrose by means of invertase gave values about one-tenth those of the reducing sugar, and these values showed small fluctuations of doubtful significance during growth and inhibition. No appreciable amounts of starch were present either before or after growth. Dextrins were present, if at all, only in minute amounts.

The role of phosphorylated intermediates was next considered. Cold water extracts of the inhibited sections contained inorganic phosphorus equivalent to 0.0099 per cent. of the fresh weight. After acid or alkaline hydrolysis under varying conditions, including up to three hours' boiling with 1 NH_2SO_4, the figure was increased only to 0.0107 per cent. of the fresh weight, i.e., only by eight per cent. These values are all extremely small and indicate that there is no appreciable content of phosphorylated intermediates after growth inhibition. It should be added that the method gave satisfactory results when tested with pure glucose-1- and glucose-6-phosphate.

It is evident, therefore, that the growth inhibitors cause a real disappearance of hexose residues, and that this is parallel, but not proportional to, the inhibition of growth. The growth inhibition is not itself *due* to disappearance of reducing sugar, since many experiments in which various carbohydrates were added to the solutions gave no increase in growth. It must be due rather to changes in metabolism. The following experiments show that the reducing sugar which has disappeared is not consumed in respiration. The oxygen consumption of the sections was determined in the usual way by the use of Warburg respirometers in the minimum of weak red light. Since approximately one hour was consumed in the prep-

TABLE II

GROWTH AND OXYGEN CONSUMPTION OF 15 MM. SECTIONS OF ETIOLATED PEA STEMS AFTER 2 HRS. AND AFTER 12 HRS. IN SOLUTION

	GROWTH AS PER CENT. ELONGATION	OXYGEN CONSUMPTION (Q_{O_2})		RATIO Q_{O_2} IN INHIBITOR: Q_{O_2} IN AUXIN CONTROL (AFTER 2 HRS.)
		AFTER 2 HRS.	AFTER 12 HRS	
In water	20	5.52	3.34	.....
In auxin (1 mg./l.)	51	6.36	3.82	.....
In auxin plus:				
Iodoacetate 6×10^{-4} M	26	4.69	3.32	0.74
Arsenite 1×10^{-4} M	26	5.51	2.89	0.87
Fluoride 5×10^{-3} M	25	6.18	4.47	0.97

aration, the first figures were obtained two hours after cutting and thereafter the respiration was followed for 12 hours. This corresponds to the period in which virtually all growth is completed. The results are presented in table II.

The following conclusions can be drawn: *first:* the respiration falls off steadily with time in all solutions; *second:* auxin (indoleacetic acid, 1 mg./l.) causes a definite increase in respiration rate, amounting to 15%, both after two and after 12 hours; *third:* the three inhibitors, at concentrations producing a growth reduction of one half, all lower the respiration rate at first. With iodoacetate and arsenite the rate remains depressed throughout the period of measurement, but with fluoride the final rate is higher than that of the controls. This parallels the data in the last column of table I which show that for fluoride the fall in reducing sugar is greater than for the other inhibitors.

The direct effect of auxin in accelerating respiration is of interest since under similar conditions (freshly cut sections, in water alone) no such effect is found on the Avena coleoptile. Only by pre-soaking coleoptile sections in sucrose or malate could an acceleration of respiration by auxin be es-

tablished [COMMONER and THIMANN (2); BERGER, SMITH and AVERY (1)]. The increase was obtained in all our experiments and its magnitude varied between 14 and 20%.

In regard to the action of the inhibitors, it is evident from table II that, at least with iodoacetate and arsenite, the inhibitor does not promote the destruction of sugar by respiration. In view of the arguments above, it must therefore be concluded that these inhibitors cause the conversion of reducing sugar to substances of a non-carbohydrate nature. It is hoped that more detailed study of these processes will shed light on the role of auxin itself in controlling growth.

HARVARD BIOLOGICAL LABORATORIES
CAMBRIDGE, MASSACHUSETTS

LITERATURE CITED

1. BERGER, J., SMITH, P., and AVERY, G. S., JR. The influence of auxin on respiration of the Avena coleoptile. Amer. Jour. Bot. **33**: 601–604. 1946.
2. COMMONER, B., and THIMANN, K. V. On the relation between growth and respiration in the Avena coleoptile. Jour. Gen. Physiol. **24**: 279–296. 1941.
3. SHAFFER, P. A. and SOMOGYI, M. Copper-iodometric reagents for sugar determination. J. Biol. Chem. **100**: 695–713. 1933.
4. THIMANN, K. V., and BONNER, W. D., JR. Experiments on the growth and inhibition of isolated plant parts. I. The action of iodoacetate and organic acids on the Avena coleoptile. Amer. Jour. Bot. **35**: 271–281. 1948.

NOTES

SEVENTIETH ANNIVERSARY OF

CHARLES ALBERT SHULL

JANUARY 19, 1949

The American Society of Plant Physiologists conveys its cordial birthday greetings to Dr. Charles A. Shull on the occasion of his seventieth anniversary. It is a pleasant privilege for PLANT PHYSIOLOGY as the official journal of the Society to serve as the medium of communicating the organization's felicitations to its first president and first editor-in-chief. The Shulls continue to reside in Asheville, North Carolina, where they have been since his retirement in 1944. Dr. Shull continues to take an active interest in the affairs of the American Society of Plant Physiologists, having been reappointed chairman of the Finance Committee at the recent Cincinnati meeting. We are pleased to extend an expression of appreciation to Dr. Shull for his extensive period of service to the Society and the science of plant physiology in general.

Cincinnati Meeting.—The twenty-third annual meeting of the American Society of Plant Physiologists, September 8 to 10, 1948, was well attended even though it was the first meeting of the Society held independently of the large A.A.A.S. conventions. The horticulturists met at the same time and place and the usual fine spirit of cooperation and fellowship between the groups was much in evidence. The program comprised 45 submitted papers and three symposia. One of these on Enzymes in Relation to Growth and Metabolism included H. K. Mitchell, R. H. Burris, C. R. Dawson, and James Bonner as speakers. The two Symposia on Growth Substances and Selective Herbicides were presented jointly by the A.S.H.S. and A.S.P.P. A. J. Haagen-Smit, Folke Skoog, and J. W. Mitchell discussed the chemistry, organ formation and correlations, and plant responses to synthetic compounds, with F. W. Went as discussion leader on the symposium on Growth Substances. Participants in the herbicide symposium were R. Blondeau and J. van Overbeek. W. H. Minshall, C. L. Hamner, R. D. Sweet, K. C. Barrons were speakers on the herbicide program.

There were 146 in attendance at the Plant Physiologists' Dinner with Dr. D. B. Anderson presiding. Dr. F. W. Went, the retiring president, gave a stimulating address on "The Bright Future of Plant Physiology." By virtue of a gift to the Society for an additional Stephen Hales Award, the names of Robert Emerson and David R. Goddard were announced as the dual recipients of Hales Awards. Dr. Emerson was honored for his outstanding contributions in the field of photosynthesis, and Dr Goddard merited the award on the basis of his excellent work on respiration. The

name of Henry H. Dixon, Trinity College, Dublin, Ireland was announced as the recipient of the Charles Reid Barnes Life Membership Award. Dr. C. S. French was appointed by the Executive Committee as a new member of the Editorial Board of PLANT PHYSIOLOGY.

At the business meeting of the Society, the report of the Editor-in-Chief emphasized the tremendous increase in the cost of printing the official journal. This cost has increased $2,000 a year each year for the past three years. The report of the Executive Secretary-Treasurer also emphasized the effect of the increased financial burdens facing the Society. Both reports set forth the need for increased revenue to the Society in order to maintain its present financial statue. A discussion developed as to where the Society would meet in 1949. Plans have been initiated for the Society to meet with the A.A.A.S. during the Christmas holidays of 1949.

Stephen Hales Awards.—The American Society of Plant Physiologists awarded the Stephen Hales' prizes for 1948 to Dr. Robert Emerson and Dr. David R. Goddard. Dr. Emerson is best known for his contributions to the field of photosynthesis. His investigations of the effects of flashing light upon photosynthetic rates and of the quantum efficiency of the photochemical process represent important milestones in the progress of plant science. Plant physiology has benefited greatly from the critical and incisive approach that has characterized Dr. Emerson's work on his chosen subject.

Dr. Goddard's studies of the comparative respiratory physiology of plant tissues and of the interrelationships of plant respiration and fermentation are unique. With great skill and ingenuity he solved the problem of identifying cytochrome and cytochrome oxidase, and of isolating these two catalysts from wheat germ. His enthusiastic personality and broad biological interests are reflected in his unusual success as a teacher at both the elementary and advanced levels of plant science.

Charles Reid Barnes Honorary Life Member.—Henry Horatio Dixon was born in Dublin, Ireland, in 1869, to George and Rebecca (Yeates) Dixon. He received his education at Rathmines School and Trinity College in Dublin and at the University of Bonn. His achievements have been many and include Classical Scholar, 1891; First Senior Moderator in Natural Science, 1892; Assistant to the Professor of Botany at Dublin University, 1892–1904; Professor of Botany at the University of Dublin since 1904; Professor of Biology at Trinity College of Dublin since 1922. In addition, he has acted as Director of the Trinity College Botanical Garden and Keeper of the Herbarium, and Trustee of the National Library of Ireland.

Numerous honors have been bestowed upon Dr. H. H. Dixon. He is a member of both the Royal Society of Dublin and of London, and a corresponding member of the American Society of Plant Physiologists. Other honoraria include membership on the Council of the Royal Dublin Society;

the Managing Committee of the Imperial Bureau of Mycology; the Council of the International Institute of Agriculture and Chairman of the Commission on Biochemistry. He has served as Commissioner of Irish Lights; Visiting Professor at the University of California; President of the Royal Dublin Society; Vice-President of the Physiological Section of the International Botanical Congress at Cambridge in 1930; Honorary Chairman of the Sixth International Botanical Congress at Amsterdam in 1935; Croonian Lecturer before the Royal Society of London in 1937. He was awarded the Boyle Medal of the Royal Dublin Society in 1917 and now, our Charles Reid Barnes Life Membership. Professor Dixon has published many scientific papers especially in the Proceedings of the Royal Societies of Dublin and London. His books include "Transpiration and the Ascent of Sap" (1914); "Practical Plant Biology" (1932, 1943); "The Transpiration Stream" (1924).

Diagnostic Techniques for Soils and Crops.—Edited by H. B. Kitchen. American Potash Institute, Washington, D. C. 308 pages. 1948. $2.00.

The authors of this book, all eminent scientists in their respective fields, have brought together in one volume much of what is now known about diagnosis of soils and crops for optimum production. Various methods of determining the fertility status of soils and nutritional requirements of crops are comprehensively described, compared and evaluated. The book gives a critical appraisal of important phases of the recent rapid progress in determining nutrient needs and describes appropriate applications and complementary uses of various procedures.

The contents comprise the following sections: Historical Introduction, by Firman E. Bear; Chemical Methods for Assessing Soil Fertility, by Dr. Michael Peech; Correlation of Soil Tests with Crop Response to Added Fertilizers and with Fertilizer Requirement, by Dr. H. Bray; Operation of a State Soil-Testing Service, by Dr. Ivan E. Miles and Dr. J. Fielding Reed; Operation of an Industrial Service Laboratory for Analyzing Soil and Plant Samples, by Dr. Jackson B. Hester; Plant-Tissue Tests as a Tool in Agronomic Research, by Dr. Bert A. Krantz, Dr. Werner L. Nelson, and Dr. Leland F. Burkhart; Plant Analysis—Methods and Interpretation of Results, by Albert Ulrich; Biological Methods of Determining Nutrients in Soils, by Dr. S. C. Vandecaveye; Visual Symptoms of Malnutrition in Plants, by Dr. J. E. McMurtrey, Jr.

The book is $7\frac{1}{4}'' \times 10\frac{1}{4}''$, is bound in durable green cloth and the illustrations, of which all are highly informative and of best professional quality, include 5 color plates, 36 halftones, and 26 drawings.

This publication is a major contribution in its field. The authors and the corporate sponsor of the project are to be commended for its timeliness, excellence and the modest price of the book.

Bibliography of the Literature on the Minor Elements and their Relation to Plant and Animal Nutrition.—Fourth Edition, Volume I, 1948.

HERBERT C. BREWER, Director Chilean Nitrate Educational Bureau, Inc., 120 Broadway, New York 5, New York. 1037 pages.

This is the fourth edition, volume 1 of the Bibliography of the Literature on the Minor Elements and their Relation to Plant and Animal Nutrition. It is the eleventh publication in the series which began with the first edition in 1935. This edition contains approximately 10,000 abstracts and is intended primarily for the use of institutions and scientists engaged in agricultural and nutritional research. Interest in minor element reference literature is world-wide and continues to expand. Demand for the bibliography has increased correspondingly and consequently supply of some earlier volumes is now exhausted and complete sets thereof are no longer available. This edition, however, includes all material published in the eight previous volumes together with many new references. It may be considered, therefore, to be complete as of June 30, 1947.

Plant and animal scientists will feel a deep sense of gratitude to Mr. Herbert C. Brewer and his associates of the Chilean Educational Bureau for the great service to individual investigators and to the general advancement of scientific knowledge in the preparation of this costly and extremely comprehensive series of abstracts. This bibliography may well be considered an indispensable reference text for anyone working in the field of plant and animal nutrition. The coverage is world-wide and the abstracts are succinct and accurate. The volume contains four separate indices which add immensely to its usefulness and which facilitate ready and rapid use of the bibliography. The indices comprise an author index, element index, general nutrition index and a botanical index, all of which are quite comprehensive and cross-referenced.

Bibliography of the Literature on Sodium and Iodine in Relation to Plant and Animal Nutrition.—First Edition, 1948. HERBERT C. BREWER, Director Chilean Nitrate Educational Bureau, Inc., 120 Broadway, New York, New York. 123 pages.

Relatively little is known of the functions of sodium and iodine in reference to the nutritive qualities of food and feed crops. The indications of positive values are too strong to be ignored indefinitely, however, and this bibliography has been prepared in anticipation of a substantial development of interest in this field of research. This bibliography represents a very comprehensive undertaking on the part of the Chilean Nitrate Educational Bureau and it will render a very important service to plant and animal scientists. The present volume endeavors to review papers published just prior to and during the war. The publishers plan to review the more recent papers in early additional publications.

This publication employs a two-column format giving each review by subject, title, author, journal of original publication, and abstract journals in which reviews of the paper may be found. The indices include the usual alphabetical author index and a supplemental index of elements sub-

indexed with an alphabetical list of authors whose papers deal with a particular element.

There is also a botanical index giving the alphabetical list of plants and the elements in relation to each of these and a concluding index on general nutrition in which the animal species are alphabetically arranged with sub-headings under each species describing the elements discussed in the papers reviewed.

Vernalization and Photoperiodism: A Symposium.—A. E. MURNEEK and R. C. WHYTE *et al.* Chronica Botanica Company, Waltham 5, Massachusetts. 196 pages. 1948. $4.50.

This important publication is a comprehensive and critical compilation of important researches on vernalization and photoperiodism and related phenomena of plant development. Various phases of the subject are treated in fifteen sections, each by a specialist in the particular field. These sections comprise a foreword by K. V. Thimann; history of research in vernalization by R. C. Whyte; history of research in photoperiodism by A. E. Murneek; hormones in relation to vernalization and photoperiodism by Karl C. Hamner; wave-length dependence and the nature of photoperiodism by H. A. Borthwick, M. W. Parker and S. B. Hendricks; photoperiodicity of flowering under short day with supplemental light of different wave lengths by G. L. Funke; nutrition and metabolism as related to photoperiodism by A. E. Murneek; anatomical and histological changes in relation to vernalization and photoperiodism by R. H. Roberts and B. E. Struckmeyer; length of day in the climates of past geological eras and its possible effects upon changes in plant life by H. A. Allard; vernalization and photoperiodism in the tropics by S. M. Sircar; preliminary observations of phenological data as a tool in the study of photoperiodic and thermal requirements of various plant material by M. Y. Nuttonson; thermoperiodicty by F. W. Went; studies on photoperiodicty in the tropics by Erwin Bünning; the developmental physiological meaning of the endogenous daily rhythm in plants by Erwin Bünning; and contributions to the genetics of photoperiodism by A. Lang.

The discussion includes large amounts of original data from various sources which are very logically co-ordinated and carefully evaluated by each of the authors. The book is liberally illustrated, containing excellent photographs and tables. The format is excellent; the type is large and the style and arrangement of contents are adapted to rapid reading and ready reference. The book concludes with very comprehensive, separate author and subject indices.

Annual Review of Biochemistry.—Edited by J. MURRY LUCK. Vol. XVII, 1948. Annual Reviews, Inc., Stanford, California. 801 pages. $6.00.

The 1947 volume of this well known review series announces changes in editorial policy which envisage new and separate reviews in special fields

such as plant physiology, physical chemistry, and medicine, respectively. An accelerated rate of publication of scientific papers in all fields seems to indicate the desirability of establishing new review series rather than to seriously curtail output by undue brevity or complete omission of certain papers in the existing reviews under the direction of Annual Reviews, Inc. This volume carries twenty-seven sections of which the following will be of special interest to plant scientists: biological oxidations and reductions by H. Weil-Malherbe; non oxidative enzymes by J. B. Sumner; chemistry of the carbohydrates by D. A. Prins and R. W. Jeanloz; x-ray crystallographic studies of compounds of biochemical interest by D. Crowfoot; the chemistry of the proteins and amino acids by K. O. Pedersen; nucleoproteins, nucleic acids, and related substances by E. Chargaff and E. Vischer; carbohydrate metabolism by B. Vennesland; the metabolism of proteins and amino acids, by J. B. Allison; the vitamins by B. L. Oser; biochemistry of the natural pigments by E. Lederer; the terpenes by N. T. Mirov; photosynthesis by E. C. Wassink; mineral nutrition of plants by H. Burström; plant hormones by P. W. Zimmerman and A. E. Hitchcock; bacterial metabolism by I. C. Gunsalus; physiological aspects of genetics by G. W. Beadle. The volume contains a comprehensive subject and author indices.

Vegetatio Acta Geobotanica.—Edited by G. Braun-Blanquet. W. Junk Publishers, The Hague, 13 van Stolkweg, Netherlands. Vol. 1, No. 1. 30 Dutch guilders per volume. 1948.

This new publication is the official organ of the International Association of Phyto-sociology. The publication will be of interest to ecologists and physiologists as well as to specialists in conservation and reclamation. Some papers will also appeal to agronomists and economic botanists. The journal also intends to be a value to sylviculturists and in the field of soil science. Papers in the first number appear both in the French and English language. The format is attractive and permits rapid scrutiny. Tables are well arranged and the photographs and charts are excellently reproduced. The form of literature citations permits rapid reference.

Hydrobiologia: Acta Hydrobiologica, Limnologia et Protistologica.—Dr. van Oye, Secretary. W. Junk Publishers, 13 van Stolkweg, The Hague, Netherlands. Vol. 1. 1948. 40 Dutch guilders.

Hydrobiologia will publish articles embodying research in the field of hydrobiology, limnology and protistology. It will include investigations in the field of plant and animal biology, marine and fresh water forms, as well as research and systematic botany and taxonomy of the groups covered. The journal will also contain reviews of recent books and papers. Nine well-known scientists constitute the editorial board on which Dr. W. K. Taylor of the University of Michigan, Ann Arbor, Michigan, is the American representative.

Advances in Enzymology and Related Subjects of Biochemistry.—Volume VIII, 1948, edited by F. F. Nord. Interscience Publishers, Inc., 215 Fourth Avenue, New York 3, N. Y. 538 pages. $8.00.

Of the ten sections in the current volume of this well known series, the following will be of particular interest to plant scientists: Functioning of the Cytoplasm by Ludwik Monne; Dehydropeptidases by Jesse P. Greenstein; Alkaloid Biogenesis by Ray F. Dawson; Certain Aspects of the Microbiological Degradation of Cellulose by F. F. Nord and J. C. Vitucci; Synthesis of Lipides by A. Kleinzeller; The Biochemistry of Fatty Acid Catabolism by F. L. Breusch; and Lipoxidase and the Autoxidation of Unsaturated Fatty Acid by Sune Bergström and Ralph T. Holman. The current volume contains both author and subject indices and also a cumulative index for volumes one to eight inclusive.

Submicroscopic Morphology of Protoplasm and its Derivatives.—A. Frey-Wyssling. Elsevier Publishing Company, Inc., 215 Fourth Avenue, New York 16, New York. 255 pages. 1948. $6.00.

This volume printed in English is actually a second edition of Frey-Wyssling's "Submicroskopische Morphologie des Protoplasmas und seiner Derivate," published in 1938 by Gebrüder Borntraeger, Berlin. Physical and chemical problems associated with the minute structure of such systems as gels, vacuoles, chloroplasts, cell walls, cutin, chitin, starches and proteins, among several others, are treated in outline form, with frequent lucid section and chapter summaries. The section covering the fundamentals of submicroscopic morphology is brought up-to-date with several new tables and photomicrographs and a discussion of electron microscopy, polarization microscopy, and other techniques utilized during the past decade. The fine structure of protoplasm and its derivatives is approached on the molecular and micellar levels, in a dimensional realm between one micron and one Ångstrom unit. In this sense, and in other ways, the author attempts to bring together some of the phenomena that bridge the gaps between descriptive morphology, at the limit of the resolving power of a microscope, and cellular physiology.

The outline manner of presentation makes it possible to include a vast amount of factual information. Most of this information is well documented with citations to the original literature and made readily intelligible by means of clearly constructed graphs and diagrams.

Skillfully woven into the text are discussions of some of the current theoretical interpretations of structural phenomena concerned with the nature of protoplasm, such as the micellar theory and its modifications, the chromonemia theory, and the fibrillar hypothesis of chromosome structure. Some of the interpretations seem to be purposefully over-simplified in the author's sincere effort to indicate the bearing of factual material on broad biological problems of protoplasmic structure, and to clarify these problems for specialists in other fields. The inclusion of 715 refer-

ences or approximately twice as many as in the original is evidence of the extensiveness of revision in the second edition.

Commercial Flower Forcing.—Alex Laurie and D. C. Kiplinger. Fifth edition, 1948. The Blakiston Company, Philadelphia, Penn. 550 pages. $4.75.

This book covers fundamentals and their practical application to the culture of greenhouse crops. It is a new edition of a very successful text and guide to commercial flower forcing and it presents many important new developments of the most recent origin. Every phase of the subject has been brought up to date from greenhouse construction to costs of production and marketing. The book covers the methods of gravel culture and controlled nutrition, control of tests by fumigants and aerosols and the utilization of photoperiodism in the production of ornamentals. Separate chapters are devoted to the status and development of the industry; greenhouse construction and heating; factors influencing plant growth; soils; growing ornamentals in gravel culture; fertilizers; reproduction; diagnosing greenhouse ills; major and minor crops; miscellaneous cut-flower crops; bulbs corms and tubers; flowering pot plants; foliage plants; forcing hardy plants; cloth houses; wholesale marketing; and costs of production. The format of the book and the style of writing permit rapid perusal. The text is abundantly illustrated and the photographic reproductions are of high quality. The book concludes with a very comprehensive cross-reference index of 13 pages.

Biology of Pathogenic Fungi.—Edited by Walter J. Nickerson. Chronica Botanica Company, Waltham, Massachusetts. 236 pages. $5.00.

This book is a cooperative volume covering many aspects of the biology, physiology, and biochemistry of the fungi pathogenic for man. It is not a clincial manual nor a compendium on all the fungi of medical importance, but presents information and viewpoints from biology and chemistry generally bearing on these difficult forms. It aims to bring together facts and interpretations for purposes of comparison with other micro-organisms and to further investigations of both theortical and practical nature on the fungi pathogenic for man. The implications for disease control are stressed.

The book includes twelve chapters contributed by the editor and twelve collaborators. The fungi discussed are primarily the human pathogens; others are mentioned only incidentally. Though some of the chapters are scarcely or not at all concerned with physiology, that phase is quite definitely stressed in the book as a whole. A brief introduction by the editor is followed by chapters on the parasitic Torulopsidoideae (Lodder and de Minjer), chromoblastomycosis (Carrion and Silva), *Pityosporium* (Benham), *Coccidioides* (Emmons), survey of recent work in Italy (Ciferri and Redaelli), the action of sulfonamides and antibiotic agents (Wolf), geographic distribution (Martin), nutrition and metabolism (Nickerson

and Williams), metabolic products (Nickerson), lipids of fungi (Peck) and respiration and fermentation (Nickerson). The last four chapters, with the seventh, constitute a valuable summary of recent physiological work on human pathogenic fungi. Each chapter has its separate bibliography but these are bound together by the cumulative indexes to authors, organisms and subjects. The volume as a whole presents in accessible form a large amount of information of value to all who are concerned with the physiology of the fungi or are working on the species which cause human disease.

The Scientific Paper.—S. F. Trelease. Williams and Wilkins Company, Baltimore, Maryland. 152 pages. 1947. $2.00.

Originally published under the title "The Preparation of Scientific and Technical Papers," this manual is intended to help students and scientists who are preparing papers on scientific and technical subjects. In its present form, it has been throughly revised and almost completely rewritten. The directions given are meant principally for the preparation of a thesis or dissertation, but apply equally well to the writing of papers in the fields of agriculture, engineering, medicine and other branches of scientific and technical endeavor. A new feature is the section on the use of the scientific library for research purposes. Coverage in the manual is complete, from the first planning and outlining of the paper, with suggestions on subject matter and arrangement, to the physical aspects such as type sizes, kinds of illustrations, the finished form of the manuscript, and proof-reading.

This book contains a wealth of information in compact and ready reference form, including the multitudinous details which so often perplex authors, both young and old, in preparation of printer's copy designed to avoid the most common editorial criticisms. Its bibliography of more extensive and specialized texts as well as its comprehensive index contribute considerably to the value of the volume. It is a genuinely useful handbook for students and research workers in all branches of science.

Symposium on Mineral Nutrition of Plants.—Under the joint auspices of several plant science societies and industrial organizations, a symposium on Mineral Nutrition will be held at the University of Wisconsin, Madison, September 1 to 3, 1949. This symposium will be followed on September 5 to 7, 1949 by another on Plant Growth Substances under auspices of the University of Wisconsin as part of its centennial celebration.

The symposium on Mineral Nutrition of Plants is divided into five sections: 1. Nutritional status of the main soil groups of the United States. 2. Physico-chemical factors affecting nutrient availability in selected soil types. 3. Mechanism of the entry and translocation of nutrients into plant roots. 4. Role of the various nutrients in the metabolic processes within plants. 5. Modifying influences of various environmental factors upon mineral metabolism.

The nutrition symposium is under the general chairmanship of Dr. Emil

Truog, Soils Department of the University of Wisconsin. Participating organizations and their representatives are: American Society of Horticultural Science, Damon Boynton; Fertilizer Section, American Chemical Society, Jackson B. Hester; Plant Food Research Committee, The National Fertilizer Association, David D. Long; Plant Food Research Committee, The National Fertilizer Association, Malcolm H. McVickar; Chemical Corps of U. S. Army, A. G. Norman; Botanical Society of America, Robert A. Steinberg; Soil Science of America, E. Truog; American Society of Agronomy, G. W. Volk, American Society of Plant Physiology, Cecil H. Wadleigh and W. F. Loehwing; and Smithsonian Institution of Washington, D. C., R. B. Withrow.

Several invitational papers will be presented in each of the sections by specialists in the various fields. Facilities for housing and messing as well as for evening and recreational sessions are being arranged. It is hoped that the completed program can be announced early in 1949.

Scientists and Reserve Officers.—The Department of the Army has established a program of particular interest to plant physiologists and other scientists who hold Reserve commissions in the Army, and who are professionally engaged in teaching or research and development. The objectives of the program are to: (1) maintain the useful affiliation of plant physiologists and other scientists with the Organized Reserve Corps, (2) provide peacetime Reserve assignments for these officers, enabling optimum utilization of their education, experience and skills, (3) furnish mobilization assignments which will fully utilize their talents, and (4) adequately prepare these officers for mobilization.

The Technical Services of the Department of the Army submit to these Research and Development Reserve Groups research problems and projects which pose an intellectual challenge to members of the group. Thus, the program provides members of each group a type of training which is in keeping with their scientific and technical interests and competence, rather than a traditional kind of training session in which scientists have little or no interest. The program is now being implemented only in those areas where there is a definite local interest. To date, eighteen Research and Development Reserve groups have been organized. Twelve additional groups are in process of organization. Others are in the initial stages of formation. Several of these groups have been formed in communities in which large universities, industrial research laboratories, or private research foundations are located.

Provision is made to submit research projects of interest to all categories of scientists—chemists, physicists, engineers, geologists, geographers, psychologists, mathematicians, and all of the biological scientists which will include plant physiologists. Reserve officers who are currently engaged in civilian research, college or university teaching, or industrial research or development, or who in the past have had specific research experience are

eligible to make application for assignment to an Organized Reserve Research and Development Group. A group may be organized in any locality where there are twenty (20) or more qualified officer scientists who desire to participate in the program. A subgroup may be organized with ten (10) qualified members.

The program is under the general direction of the Research and Development Group, Logistics Division, General Staff, United States Army. The entire program is outlined in Department of the Army Circular Number 127, dated May 5, 1948. Inquiry about organization of an Organized Reserve Research and Development Group or about assignment to a group already organized should be made of the Unit Instructor, ORC, or of the Senior Army Instructor, ORC, in the locality in which the officer resides. In localities in which a group has already been organized, the Commanding Officer of the group will consider applications for assignment of additional officers.

PREPARATION OF MANUSCRIPTS FOR PUBLICATION IN PLANT PHYSIOLOGY

The following suggestions are made to authors writing papers for this journal in order to minimize revisions and editorial corrections. Though many points may appear arbitrary or trivial, attention to details is necessary to secure uniformity of format and style. The membership dues and subscription fees go almost entirely to publication costs, as all of our editors give their service without financial recompense. Hence the cooperation of authors in preparation of manuscripts minimizes expense and permits greater liberality as to length and number of papers published. Authors will find the following concise publications useful in preparation of manuscript copy: A. J. Riker, The Preparation of Manuscripts for Phytopathology. Phytopathology XXXVI, pages 953–977, 1946; and S. F. Trelease, The Scientific Paper: How to Prepare It. Williams and Wilkins Company, Baltimore, Maryland; 1947.

1. **Length.** The allowable length of papers is 20 printed pages, with tables and figures not to exceed 25 per cent of the total. Authors will be billed at cost for overrun and excess of figures and tables.

2. **Format.** In the preparation of typescripts, authors are requested to examine a printed copy of the journal of PLANT PHYSIOLOGY to note the position of headings, general organization, punctuation, abbreviations, and the methods of citing literature particularly, in order to bring the script into conformity with the general style of the journal. Leave a 3-inch margin at the top of the first page, liberal side margins, and double spacing of lines to permit proofmarking and required editorial changes.

3. **Headings.** Leave all headings unscored and scientific names without underlining, as the editors will proofmark these items in accordance with the practice of the journal. Generic names used alone are usually capitalized but not italicized. In general, do not underscore any lines.

4. **Footnotes.** Avoid footnotes as far as possible by placing such items under acknowledgments or at end of summary. When footnotes are used, they should be numbered with Arabic numerals.

5. **Figures.** Copy for figures (photographs and drawings) should not exceed $8\frac{1}{2} \times 11$ inches. Author's name and figure number must be written on the back of copy for each figure. Do *not* put legends into or on the figure, either front or back. Type all legends *double* spaced, properly numbered to correspond to the figures, on a *separate sheet* of your manuscript. Graphs and drawings should be inked with fairly heavy lines and with lettering of approximately uniform size to insure clarity of printed figures after reduction. Photographic prints should be submitted on gloss-finish paper. Limit copy to desired visual details, trim off irrelevant foreground and minimize blank space, as charges for cuts are based on area. Unless otherwise directed, the editor will proofmark copy to determine actual size of printed figures. Old cuts will be sent to authors after printing for cost of postage if printer is so directed when galley proof is submitted.

6. **Tables.** Place each table on a separate page; do not crowd too much material into a single table. Note size of printed page (width and length, $4\frac{1}{2} \times 7$ inches) and gauge tables accordingly, leaving ample space in margins and in the interior for editorial marks. If at all possible, arrange tabular data horizontally on the page in order to obviate tilting of printed table. Use horizontal lines (rules) at top and bottom, but omit or employ such rules sparingly in interior of tables. Use vertical rules between columns. Rules and spaces (leads) are usually used in the final printed tables to separate sets of data. For footnotes to tables use the asterisk, dagger, double dagger, and section in this sequence as needed. Tabular material should be used sparingly because of its high cost.

7. **Acknowledgements.** Place acknowledgements at the end of the paper just below summary.

8. **Address.** The name of your institution and its address should also be given at the end of the summary.

9. **Literature Cited.** Use separate pages for the literature cited and type *double spaced*. Give complete citations, author, title, journal name, volume number, inclusive pages, and year. (See any literature list in a recent issue of the journal for examples.) Punctuate according to the examples, and leave no citations incomplete. Write out names of states in full. Note proper abbreviations of journals. An error frequently noted in manuscripts with long bibliographies is failure to mention in the text all citations listed. Authors are requested to check all citations in the bibliography by careful proofreading of manuscripts prior to submission.

10. **Formulae.** Use mathematical formulae only when necessary, and be conservative in the use of space.

11. **Galley Proof.** Correct all errors clearly and return galley promptly to editor. Authors will be billed at cost for alterations in the

galley proofs, other than corrections of printer's errors, involving excessive changes from manuscript copy.

12. **Mailing.** Send original copy of manuscript to editor, W. F. Loehwing, University of Iowa, Iowa City, Iowa. Retain a carbon copy to insure against loss, and to consult in case of need. Prompt return of revised manuscript copy and galley proof to editor greatly expedites publication. Write across top of title page of manuscript directions for mailing of proof, thus: Mail proof to (author's name and address). If authors wish to have original manuscripts or copy for illustration returned subsequent to publication, the editor should be so informed at the time proof is returned.

13. **Reprint Order.** These should be sent to the editor with corrected galley proof. Late orders for reprints entail extra work and expense by printer for which charges must be made to author. Type is destroyed immediately after printing and hence it becomes impossible to supply reprints after publication of papers. Send Reprint Order with *corrected galley* even if formal requisition or purchase orders for institutional agencies follow later.

14. **Abstracts.** Authors are requested to submit typed abstract (blue form) when corrected galley is returned to editor who will insert proper volume and paging for use by Biological Abstracts. If authors care to type a carbon copy of abstract on plain paper, the second abstract will be sent to Chemical Abstracts or some other suitable abstract journal.

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